Erythroid bone marrow activity and red cell hemoglobinization in iron-sufficient β-thalassemia heterozygotes as reflected by soluble transferrin receptor and reticulocyte hemoglobin content. Correlation with genotypes and Hb A_2 levels

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Background and Objectives. Ferrokinetic studies and erythroid cell ultrastructural studies have indicated some degree of ineffective erythropoiesis in heterozygous β-thalassemia, although a wide case-to-case variation was observed. In this study we applied rapid biochemical and hematologic measurements to assess erythroid marrow activity (sTfR) and reticulocyte hemoglobin content (CHr) in iron-sufficient individuals with heterozygous β-thalassemia and investigated the correlation with the degree of globin polypeptide chain imbalance by comparing parameters between β-thalassemia heterozygotes with genotypes of variable severity.

Design and Methods. We studied 57 iron-sufficient adults with heterozygous β-thalassemia, divided into groups according to genotype: group A, β^{silent}-thalassemia heterozygotes, group B, β^{+}-thalassemia heterozygotes and group C, β^{0}-thalassemia heterozygotes. Twenty-one hematologically normal individuals served as controls (group D). We measured hematologic parameters including CHr with a Bayer-Advia 120 hematology analyzer. Hemoglobins were analyzed by high performance liquid chromatography, while biochemical parameters of iron status (iron, ferritin, transferrin and sTfR) were measured with chemical, luminometric and nephelometric methods.

Results. We found significant positive correlations between sTfR values for all β-thalassemia heterozygote groups when plotted against Hb A_2 and Hb F levels ($r=0.566$, $p<0.0001$ and $r=0.283$, $p<0.03$, respectively) and a significantly negative correlation between CHr and Hb A_2 values ($r=-0.790$, $p<0.00001$). These data reflect the fine association of globin polypeptide chain imbalance with erythron expansion and the greater degree of ineffective erythropoiesis in β-thalassemia heterozygotes with more severe genotypes.

Interpretation and Conclusions. This study is the first demonstration that sTfR and CHr are useful parameters for evaluating the relative severity of different genotypes in heterozygous β-thalassemia.

Key words: reticulocyte hemoglobin content, soluble transferrin receptors, β-thalassemia heterozygotes.

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Heterozygous β-thalassemia is caused by the inheritance of a single β-thalassemia allele, whether β^{0} or β^{+}. It is usually characterized by a mild anemia with hypochromic microcytic red blood cells, elevated levels of Hb A_2, variable increases of Hb F (up to 2.0%) and polypeptide globin chain biosynthesis showing an approximately 2-fold chain excess. Ineffective erythropoiesis plays a well established role in the pathophysiology of disease expression in β-thalassemia major and intermedia. Ferrokinetic studies, as well as erythroid cell ultrastructural studies have indicated some degree of ineffective erythropoiesis in heterozygous β-thalassemia, although a wide variation from case-to-case has been observed.[1,2]

The level of soluble transferrin receptors (sTfR) in the plasma has been shown to be closely related to the number of red cell precursors in the bone marrow and to provide a reliable quantitative assessment of the rate of erythropoiesis.[3] The transferrin receptor is an integral membrane protein found on the cell surface of virtually all mammalian cells. It is composed of two identical subunits held together by disulfide bridges to form a dimer of 190kDa. Each receptor subunit is able to bind one transferrin molecule with high affinity and the function of TfR is to aid the cell in the acquisition of iron from transferrin via receptor-mediated endocytosis. The number of TfR present in a cell is tightly regulated by both the iron status and the proliferative status of the cell. Regulation occurs at the level of mRNA translation and is effected by the binding of a protein (iron responsive element binding protein, IRE-BP) to special stem-loop structures (iron responsive elements, IRE) on the TfR mRNA.[4] During red cell maturation soluble truncated monomers of membrane TfR are released into the serum.

Reticulocyte hemoglobin content (CHr) is a relatively new parameter in hematologic analyses. It is a product of cellular volume and cellular hemoglobinization, and has been proposed as an early hematologic marker of functional iron deficiency in healthy individuals.[5] It has not, however, been evaluated to date as a parameter in β-thalassemia heterozygotes, nor has it been correlated with the relative severity of β-thalassemia mutations and thus the degree of β-globin chain synthesis.

In this study we applied rapid biochemical and hematologic measurements to assess erythroid marrow activity (sTfR) and reticulocyte hemoglobin content (CHr) in iron-sufficient individuals with heterozygous β-thalassemia and investigated the correlation with the

degree of globin polypeptide chain imbalance by comparing the parameters between groups of β-thalassemia heterozygotes with different severity of genotypes.

Design and Methods

The present study was performed in accordance with the Helsinki Declaration of 1964 (as amended in 1983 and 1989) and approved by the Ethics Committee of *Aghia Sophia* Children's Hospital, Athens, Greece.

Fifty-seven adult subjects (male/female 20/37) aged 18-55 years with heterozygous β-thalassemia were included in the study. All subjects had normal renal function as determined by BUN/creatinine measurements. Furthermore, no patients had acute phase-response (C-reactive protein levels <2mg/L). The subjects were divided into three groups according to their β-globin gene mutations: Group A, β^{silent}-thalassemia heterozygotes; group B, β^{+}-thalassemia heterozygotes; and group C, β^{0}-thalassemia heterozygotes. Twenty-one hematologically normal (Hb A/Hb A homozygotes; male/female: 10/11) served as controls (group D).

Blood counts were measured automatically, using the ADVIA^{R} 120 Hematology System (Bayer Corporation, Tarrytown, NY, USA) for Hb, red blood cells, hematocrit, and red cell indices. Reticulocyte measurements included percentage of reticulocytes, mean Hb concentration of reticulocytes (CHCMr) and mean cell volume (MCVr). Reticulocyte hemoglobin content (CHr) was calculated as MCVr × CHCMr.[6]

Hb A_2 and Hb F were quantified by weak cation-exchange high performance liquid chromatography (wce-HPLC) according to a procedure described elsewhere, using the β-Thalassemia Short Program on Variant Bio-Rad HPLC apparatus (Bio-Rad Laboratories, Hercules, CA, USA).[7]

Serum iron concentration was determined with the ferrozine method using the ADVIA-1650 Chemistry System (Bayer Corporation, Tarrytown, NY, USA). Serum ferritin levels were measured by a two site chemiluminescence immunoassay using an Advantage chemiluminescence autoanalyzer (Nichols Institute Diagnostics, CA, USA). Transferrin levels were measured by latex-particle-enhanced immunonephelometric assay on a BN ProSpec nephelometer (Dade Behring, Liederbach, Germany). The transferrin index (TI) was calculated as the Fe/transferrin molar ratio and represents the transferrin saturation.[8]

Soluble transferrin receptor levels were determined in sera using the *N latex sTfR* assay (Dade Behring, Liederbach, Germany).

This test is a fully mechanized latex-particle-enhanced immunonephelometric assay for the quantitative determination of sTfR in serum, which was processed on the BN ProSpec nephelometer (Dade Behring, Liederbach, Germany).[9] Polystyrene particles coated with monoclonal antibodies to human transferrin receptor were agglutinated when mixed with samples containing sTfR. The intensity of the scattered light quantified by the nephelometer depended on the sTfR content of the sample. The sTfR concentration could, therefore, be determined by reference to a standard of known concentration. A six point reference curve was constructed by automatic dilution of one single calibrator. The assay had a working range between 0.136 and 4.36 mg/L, using 1:20 diluted samples. Samples with higher sTfR concentrations were re-analyzed automatically.

Erythroid bone marrow activity for each group of β-thalassemia heterozygotes was calculated by dividing the mean sTfR value of each group (A, B or C) by that of the control group (D).

Genomic DNA was isolated from white blood cells. The α-globin genes were analyzed for deletions using Southern blot analysis and for point mutations using polymerase chain reaction (PCR)-based methods as previously described.[10] The β-thalassemia mutations were characterized using a combination of denaturing gradient gel electrophoresis (DGGE) followed by amplification refractory mutation system (ARMS)-PCR, restriction-endonuclease (RE)-PCR or direct sequencing as previously described.[11]

For statistical analysis data are expressed as mean ± SD. The correlation coefficient r between the parameters tested was computed using least squares regression analysis. The *p* values reported are two-tailed. All the statistical procedures were performed using the STATGRAFICS PLUS version 2.1 for Windows program (Graphic Software System) while the regression plots were prepared using the Sigma plot version 3.0 program (Sigma-Aldrich Chemical Co).

Results

DNA analysis of the β-globin gene revealed the following mutations: 10 samples with mild/silent β-thalassemia mutations (+1480 C→G, -101 C→T), (Group A); 26 with β^{+}-thalassemia mutations (IVSI-n110 G→A, IVSI-n6 T→C, IVSII-n745 C→G) (Group B) and 21 with β^{0}-thalassemia mutations (IVSI-n1 G→A, CD39 C→T, CD5 -CT, CD6 -A) (Group C).

Table 1 summarizes the relevant hematologic findings, including reticulocyte count and reticulocyte hemoglobin content (CHr), for the three groups of β-thalassemia heterozygotes and the normal controls. The parameters associated with iron status and erythroid marrow activity are sum-

Table 1. Hematologic data of individuals with heterozygous β-thalassemia.

	Group A silent	*Group B* β^+	*Group C* β^0	*Group D*
Hb (g/L)	135±11 (118-157)	122±12 (100-151)	112±8 (91-128)	141±10 (121-161)
MCV (fL)	88.2±5.1 (79.8-95.8)	68.5±4.8 (59.4-77.5)	66.3±3.51 (58.5-74.1)	91.2±3.7 (87.3-104.2)
MCHC (g/L)	321±10 (303-334)	307±9 (288-324)	305±12 (276-327)	333±6 (321-345)
Retics (%)	1.2±0.4 (0.7-1.8)	1.2±0.5 (0.5-2.5)	1.7±0.7 (1.0-3.9)	1.1±0.3 (0.6-1.7)
CHr (pg)	29.6±1.6 (27.0-32.0)	22.5±1.4 (19.5-24.9)	21.4±1.6 (19.5-25.3)	30.2±0.9 (28.7-32.4)
Hb A_2 (%)	3.4±0.5 (2.6-4.0)	4.9±0.6 (3.8-5.8)	5.4±0.4 (4.9- 6.7)	< 3.5
Hb F (%)	0.8±0.8 (0.7)* (0.0-2.2)	0.8±0.8 (0.6)* (0.0-2.6)	1.5±1.6 (1.2)* (0.0-6.6)	<0.5

**Median value in parentheses.*

Table 2. Iron status and erythroid bone marrow activity in individuals with heterozygous β-thalassemia.

	Group A silent	*Group B* β^+	*Group C* β^0	*Group D*
Iron (μmol/L)	16.6±4.1 (10.0-23.0)	18.4±6.0 (9.0-34.0)	18.5±6.4 (11.0-34.0)	16.1±5.3 (9.0-30.0)
Transferrin (μmol/L)	32.7±3.0 (29.0-37.0)	33.6±5.0 (27.0-44.0)	30.7±5.1 (24.0-45.0)	31.8±4.4 (25.0-39.0)
Transferrin Index	0.50±0.15 (0.28-0.74)	0.55±0.19 (0.28-0.94)	0.62±0.25 (0.28-1.06)	0.51±0.18 (0.28-0.86)
Ferritin (μg/L)	81.9±81.2 (11.0-220.0)	55.2±53.6 (13.0-205.0)	78.0±65.1 (12.0-253.0)	54.3±44.6 (19.0-150.0)
sTfR (mg/L)	1.43±0.29 (0.98-1.87)	1.77±0.28 (1.40-2.41)	2.86±0.82 (1.77-5.02)	1.23±0.19 (0.93-1.59)
Bone Marrow Activity	1.16	1.44	2.34	1.0

marized in Table 2 for all four groups.

Statistical analysis of RBC indices (MCV, MCH and MCHC) did not show significant differences between values in group B and group C or between group A and group D. The values in groups B and C were, however, significantly different from those in groups A and D (*data not shown*). There was no statistically significant difference for CHr values between groups A and D ($p > 0.2$), but values were significantly different between groups B and A or D, and between groups C and A or D ($p < 0.0001$) and even between groups B and C ($p < 0.01$).

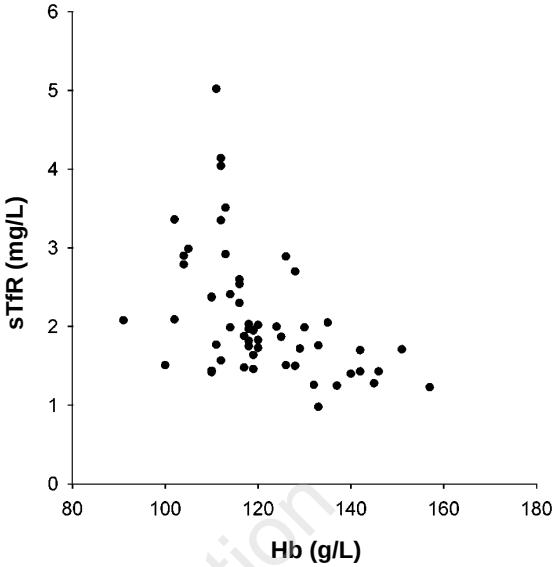

Figure 1. Correlation of sTfR levels with Hb levels in all β-thalassemia heterozygotes demonstrate an exponential decay-like regression. (sTfR = exp [2.30 – 0.01 × Hb]), r=-0.529, $p < 0.00001$).

The Kruskal-Wallis test for the parameters associated with iron status and erythroid marrow activity (Table 2) showed no significant difference between all groups for ferritin levels and transferin index (overall $p > 0.52$ and $p > 0.48$, respectively). None of the cases were iron deficient, according to the reference levels in our laboratory and those reported elsewhere.[12,13] However, sTfR levels were significantly higher in groups B and C than in groups A and D, with the highest levels compared to normal (group D) found in β^0-thalassemia heterozygotes (group C).

There was a significant negative correlation between sTfR values of all β-thalassemia heterozygote groups when these values were plotted versus hemoglobin levels (r =-0.529, $p < 0.00001$) (Figure 1). Moreover, there was a significant positive correlation between sTfR values of all β-thalassemia heterozygote groups when plotted versus Hb A_2 (r=0.566, $p<0.0001$) (Figure 2), while there was a weak but also significantly positive correlation between sTfR and Hb F levels (r=0.283, $p<0.03$).

Discussion

Heterozygous β-thalassemia is a heterogeneous disorder caused by a wide spectrum of β-globin gene mutations. These mutation apparently reduce the synthesis of β polypeptide chains to variable

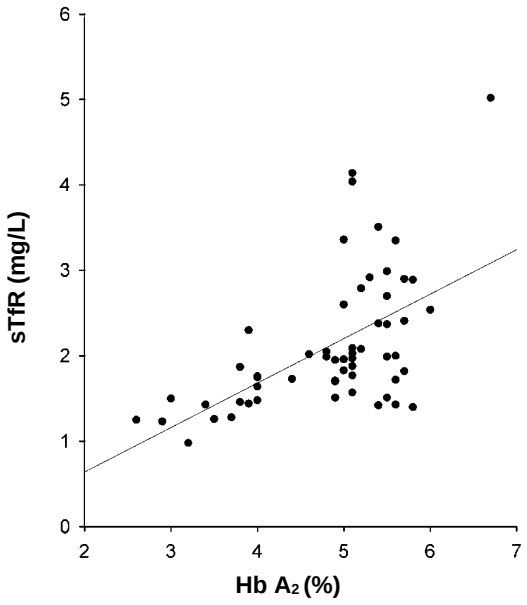

Figure 2. Correlation of sTfR levels with Hb A_2 values in all β-thalassemia heterozygotes. (sTfR = 0.52 × Hb A_2 – 0.42, r = 0.565, $p < 0.0001$).

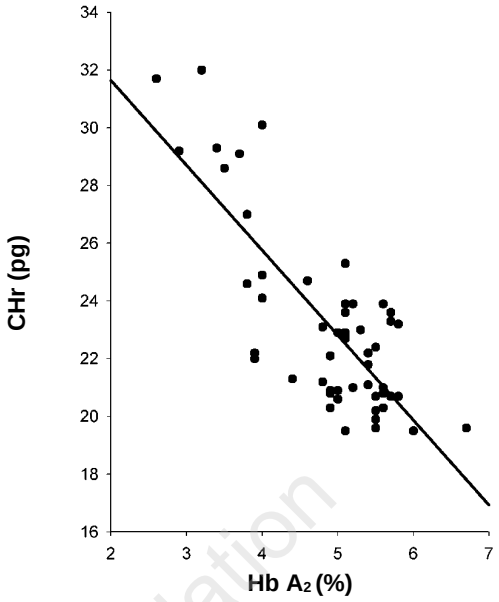

Figure 3. Correlation of CHr values with Hb A_2 values in all β-thalassemia heterozygotes. (CHr = -2.94 × Hb A_2 + 37.5, r = -0.790, $p < 0.00001$).

degrees as manifested by the severity of hematologic phenotypes in β-thalassemia heterozygotes and clinical phenotypes in β-thalassemia homozygotes.[14,15] Measuring globin chain synthesis through globin biosynthesis experiments would be a direct way to evaluate mutation severity, but results are often inconsistent, probably because of technical difficulties associated with biosynthesis methods.[16] Thus the β-thalassemia heterozygotes in this study were selected to represent examples of minimal (group A), moderate (group B) and severe (group C) reduction in β polypeptide chain synthesis based on their genotypes, with the aim of evaluating the association of the degree of globin chain imbalance with erythroid marrow activity and/or red cell hemoglobinization.

Previous studies have shown a correlation between red cell indices and severity of genotype in β-thalassemia heterozygotes.[11,14] The findings in this study are consistent with this, although differences were not statistically significant between all genotypes.

Reticulocyte hemoglobin content (CHr) is a product of cellular volume and cellular hemoglobinization. CHr has been proposed as an early hematologic marker of functional iron deficiency in otherwise healthy individuals, and as a parameter with which to monitor iron therapy,[17] although it is apparently not a useful parameter in β-thalassemia heterozygotes.[6,18] This study on iron-sufficient β-thalassemia heterozygotes (Table 2) did not find a significant difference in CHr values between patients with mild thalassemia (group A) and those without (group D), although there were significant differences for values between mild versus moderate (group B) versus severe carriers (group C). Furthermore, there was a statistically significant negative correlation between the CHr and Hb A_2 levels (Figure 3). There are several possible mechanisms which can lead to raised levels of Hb A_2 including a) a post-translational route when the formation of α-δ dimers is promoted in the presence of a relative excess of α-chains, or b) the increased transcription of the δ-globin gene in *cis* to certain β-globin gene promoter mutations because of a change in the binding of transcription factors.[19] None of the heterozygotes in this study had β-globin gene promoter mutations, and thus the correlation between Hb A_2 levels and CHr demonstrates an excellent index for the severity of the β-thalassemia mutations with the degree of red cell hemoglobinization. Furthermore, the degree of globin chain imbalance is suggested by the relatively higher sTfR levels found in β-tha-

lassemia heterozygotes with moderate and especially severe genotypes, compared to the levels in patients with mild genotypes or in normal individuals. All 3 parameters are closely related to the severity of β-thalassemia mutations, and even within group B we observed statistically significant differences for CHr, sTfR and Hb A_2 between cases heterozygous for the severe β^+ mutation IVSI-n110 G→A and the mild β^+ mutation IVSI-n6 T→C ($p < 0.04$, $p < 0.04$ and $p < 0.004$, respectively).

With the exception of a single report[20] other studies have found that the sTfR levels in β-thalassemia heterozygotes are higher than in normal non-thalassemic individuals.[21-23] This study represents the first evaluation of sTfR levels in correlation with the degree of globin chain imbalance in β-thalassemia heterozygotes, clearly demonstrating a positive correlation between the degree of globin chain imbalance and sTfR levels, and thus erythroid bone marrow activity (Table 2). Even the minimal globin chain imbalance resulting from heterozygosity of very mild (silent) β-thalassemia mutations causes ineffective erythropoiesis or possibly shortened red cell survival, which is in agreement with findings from an analogous study in α-thalassemia heterozygotes.[24]

The significant positive correlation between sTfR values for all β-thalassemia heterozygote groups when plotted against Hb A_2 ($r=0.566$, $p<0.0001$) (Figure 2), also reflects the fine association of globin chain imbalance with erythron expansion, and additionally the correlation between sTfR levels and CHr is consistent with this. Furthermore, the weak but also significantly positive correlation between sTfR and Hb F levels ($r=0.283$, $p<0.03$) shows evidence of a greater degree of ineffective erythropoiesis and F-cell selection in β-thalassemia heterozygotes with more severe genotypes.[11,25]

This study is the first demonstration that CHr and sTfR are useful parameters for evaluating the relative severity of different genotypes in patients with heterozygous β-thalassemia.

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Pre-publication Report & Outcomes of Peer Review

Contributions

CS: measurements of biochemical parameters and writing the manuscript; IP: design of the study, data analysis and editing the manuscript; JT-S: molecular analysis and editing the manuscript; HS: analysis of hemoglobins; VL: collection of samples; AM-M: measurements of hematologic parameters; AS: measurements of hematologic parameters; EK: coordinator of the study.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Dr. Carlo Brugnara, Deputy Editor. The final decision to accept this paper for publication was taken jointly by Dr. Brugnara and the Editors. Manuscript received February 13, 2003; accepted May 7, 2003.

In the following paragraphs, Dr. Brugnara summarizes the peer-review process and its outcomes.

What is already known on this topic

In heterozygote β-thalassemia, the severity of β-chain synthesis reduction and associated chain imbalance is correlated with the extent of changes in erythrocyte parameters such as MCV and MCH.

What this study adds

This study presents a careful characterization of erythrocyte, reticulocytes indices, sTfR, Hb F and Hb A_2 in iron sufficient subjects with β thal trait mutations of different severity. Changes in Hb A_2, sTfR and CHr are consistent with the severity of the genotypes.

Caveats

This study confirms what had been shown in the past with less sophisticated or less complete studies. However, it does not address the pathophysiology of the underlying disease and potential regulatory mechanisms involved. In particular, the recent report of alpha hemoglobin stabilizing protein (*AHSP; Nature 2002; 417:758, and 417:703*) provides an important novel pathway that could affect the alpha chain imbalance of β thalassemia and ultimately affect cellular characteristics and severity of the underlying genotype.