Anemia in β-thalassemia patients targets hepatic hepcidin transcript levels independently of iron metabolism genes controlling hepcidin expression

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ABSTRACT

Thalassemia associates anemia and iron overload, two opposite stimuli regulating hepcidin gene expression. We characterized hepatic hepcidin expression in 10 thalassemia major and 13 thalassemia intermedia patients. Hepcidin mRNA levels were decreased in the thalassemia intermedia group which presented both lower hemoglobin and higher plasma soluble transferrin receptor levels. There was no relationship between hepcidin mRNA levels and those of genes controlling iron metabolism, including HFE, hemojuvelin, transferrin receptor-2 and ferroportin. These results underline the role of erythropoietic activity on hepcidin decrease in thalassemic patients and suggest that mRNA modulations of other studied genes do not have a significant impact.

Key words: anemia, β-thalassemia, hepcidin, gene expression, iron overload.

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Introduction

β-thalassemia includes thalassemia intermedia (TI) and thalassemia major (TM). TM patients usually present more severe anemia requiring frequent blood transfusions compared with TI.[1] This extra iron, added to that provided by hemolysis, results in the development of secondary iron overload. In addition, despite hepatic iron overload, gastrointestinal iron hyperabsorption persists in anemic thalassemic patients, further contributing to iron burden.[2] Hepcidin, mainly synthesized by the liver,[3] is a key regulator of iron metabolism controlling iron release from reticuloendothelial and intestinal epithelial cells.[4] It is induced by inflammation[5] or iron overload.[3] By contrast, hypoxia and anemia reduce hepcidin expression and may favour iron release and availability.[6] A decrease of urinary hepcidin has been reported in thalassemic patients[7,8] and was well correlated with hepatic hepcidin mRNA expression levels.[9] Experiments on C57Bl/6 Hbb$^{th3/th3}$ mice, a murine model of thalassemia major, confirm these findings.[10] Erythropoietic activity is considered to be the main driver of hepcidin expression.[11,12] Beside hepcidin (HAMP), HFE, hemojuvelin (HJV), transferrin receptor 2 (TfR2) and ferroportin (FPN) genes play an important role in the regulation of iron metabolism. Their mutations lead to various forms of chronic iron overload in humans,[13] most of which implicate downstream hepcidin deficiency. Our aim was to evaluate the respective roles of iron load, anemia and genes controlling iron metabolism on hepcidin mRNA expression during β-thalassemia.

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Design and Methods

Patients

Twenty-three β-thalassemic patients [10 major (TM) and 13 intermedia (TI)] were compared with 10 control patients without iron overload. The study was approved by the local ethics committees and informed consent was given by patients. Control patients had undergone surgery for hepatic tumors, mainly liver metastasis. Histologic examination of their livers did not show any significant hepatic failure, fibrosis, cirrhosis or iron excess. Control hepatic samples used in this study were obtained from hepatic resection pieces and provided by the Biological Resources Centre of Rennes. Only the non-tumoral parts of the liver biopsies were used for gene expression analysis. Liver biopsies of thalassemic patients were performed for diagnostic purposes. TM patients started transfusion therapy in the first year of life and underwent different iron chelation therapies (deferoxamine or deferiprone or combined therapy). All TM and 4 TI were seropositive for hepatitis C virus. Total number of transfusions ranged from 3 to 340 units. All patients had been splenectomized.

Methods

Clinical laboratory studies

Clinical treatment and laboratory assays were routinely performed at the San Eugenio Hospital (Rome, Italy) and Ospedale Regionale per le Microcitemie, Centro Talassemici Adulti (Cagliari, Italy) for thalassemic patients, and at the University Hospital Pontchaillou (Rennes, France) for control patients. Soluble transferrin receptor (sTFR) assay was performed using IDeA® sTfR-IT assay (Orion Diagnostic, Finland).

Quantitative RT-PCR

PCR reactions were performed using qPCR-Core-kit and 18S Genomic control kit FAM-TAMRA for HAMP and MasterMix Plus for SybRGreen for FPN, HFE, HJV, TfR2, ceruloplasmin (CP), C-reactive protein (CRP) and 18S according to the manufacturer's instructions (Eurogentec®, Seraing, Belgium). Primer sequences are available upon request. The PCR was run on ABI PRISM 7000 sequence detection system (Applied Bioscience, London, United Kingdom).

Statistical analysis

The statistical analysis was performed on Statview software (SAS institute, Cary, NC, USA) using non-parametric tests including Mann-Whitney, Kruskall Wallis and Spearman. A p less than 0.05 was considered to be statistically significant.

Results and Discussion

Anemia and erythropoietic demand alter hepatic hepcidin m-RNA expression in thalassemia

In agreement with previous reports, we showed a decrease in hepcidin mRNA expression in the liver of thalassemic patients.[7,9] However, in our study, only in TI patients were hepcidin mRNA levels found to be significantly decreased compared with TM and control patients (Table 1). Iron overload was equivalent in both thalassemic groups as indicated by their similar HIC, ferritin and transferrin saturation values (Table 1) and there was no correlation between hepcidin mRNA levels and serum iron parameters. In addition, a negative correlation between hepcidin mRNA level and hepatic iron concentration was found (Figure 1A). Taken together, these data

Table 1. Clinical parameters and hepatic mRNA expression of iron metabolism genes of thalassemic and control patients.

	Control group (n=10)	Thalassemia major group (n=10)	Thalassemia intermedia group (n=13)
Sex ratio male/female	9	0.85	1
Age (years)	53 (48-78)	34.5 (23-45)	40 (21-75)
Ferritin (μg/L)	153 (37-462)	752 (209-2947)*p<0.002	657 (220-3445) *p<0.0004
HIC (μmol/gdw)	23 (3-32)	72 (16.2-321) *p<0.03	155 (3.6-410) *p<0.002
Serum iron (μmol/L)	16.4 (10.8-25.8)	40.9 (36.7-61.9) *p<0.0003	37.9 (29-45.1) *p<0.0001;**p<0.05
Transferrin saturation (%)	31 (12.7-46.9)	93 (73.2-105.2) *p<0.0003	90 (81-103.2) *p<0.0003
Soluble transferrin receptor (mg/L)	0.18 (0.112-0.301)	0.46 (0.081-0.967)	1.16 (0.279-1.85) *p<0.0001
Hemoglobin (g/dL)	14.1 (12.5-16.4)	10.2 (8.4-11) *p<0.002	8.2 (6.8-11.6) *p<0.0001; **p<0.02
Hepcidin expression (AU)	0.3 (0.02-1.18)	0.33 (0.06-2.83)	0.08 (0.01-0.85) *p<0.03; **p<0.02
Ferroportin expression (AU)	0.58 (0.13-0.95)	0.25 (0.11-0.49) *p<0.004	0.29 (0.16-1.81)
HFE expression (AU)	0.65 (0.13-0.79)	0.78 (0.44-1.35)	0.88 (0.58-1.65) *p<0.01
Hemojuvelin expression (AU)	0.48 (0.002-0.94)	0.61 (0.33-1.15)	0.52 (0.34-1.39)
Transferrin receptor 2 expression (AU)	0.66 (0.003-0.93)	0.49 (0.28-0.79)	0.48 (0.28-1.46)
Ceruloplasmin (AU)	0.84 (0.27-1.87)	0.61 (0.32-1.38)	0.67 (0.46-1.68)
C-reactive protein (AU)	0.58 (0.12-1.6)	0.11 (0.04-1.54) *p<0.03	0.15 (0.03- 0.74) *p<0.04

*Median and range are indicated for sex ratio, age, serum ferritin level, hepatic iron concentration (HIC), serum iron, transferrin saturation, soluble transferrin receptor (sTFR), hemoglobin levels and hepatic mRNA expression levels of hepcidin, ferroportin, HFE, hemojuvelin, transferrin receptor 2, ceruloplasmin and C-reactive protein genes in the different patient groups. Gene expression level was determined by quantitative RT-PCR and results were expressed in median of the ratio sample versus standard; * indicates that results are significantly different compared with control group; ** indicates that results are significantly different compared with thalassemia major group. AU: arbitrary units.*

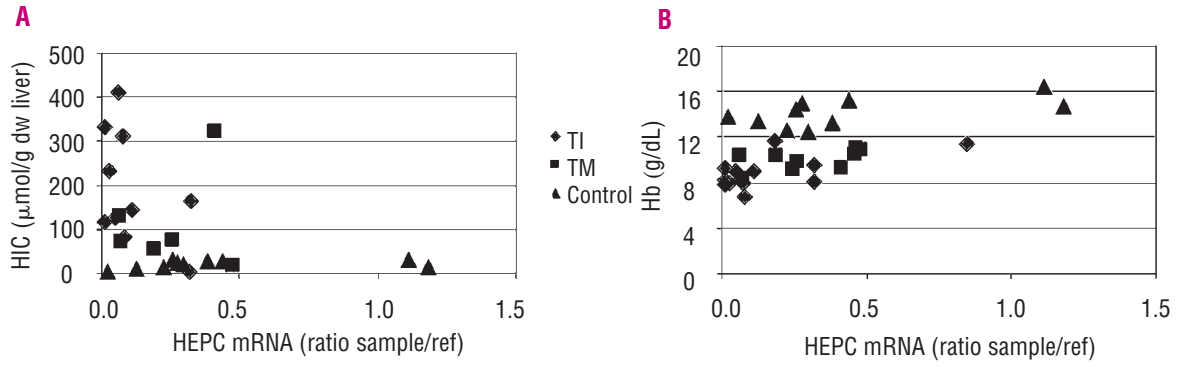

Figure 1. Relationship between hepcidin mRNA expression and biological parameters. Hepcidin mRNA levels, determined by RT-PCR, were correlated with (A) hepatic iron concentration values (HIC) and (B) hemoglobin levels (Hb). Correlation values were calculated by using the non-parametric Spearman test for the whole patient group and for the thalassemic group (black triangles for controls, squares for TM and diamonds for TI). Rho_A=0.45; p<0.03 and Rho_B=-0.551; p<0.003 for the whole patient group and Rho_A=0.5; p<0.05 and Rho_B=-0.686; p<0.002, for the thalassemic groups only.

suggest that, in thalassemia, iron overload is not a dominant signal controlling hepcidin mRNA expression. Hemoglobin levels were lower in TM than in controls and also significantly lower in TI versus TM (Table 1). They were positively correlated to hepcidin mRNA levels (Figure 1B). Furthermore, sTfR, reflecting erythropoietic activity,[14] was higher in TI than in controls (Table 1). Therefore, hemoglobin and increased erythropoietic activity status seem to be the dominant regulatory signals for hepcidin expression in thalassemia, as compared with the extent of body iron excess. This hypothesis was put forward in several recent works on mouse thalassemic models[15] and manipulated erythropoietic activity.[11,12] The potential role of GDF 15 (*Growth Differentiation Factor* 15),[16] and/or HIF (*Hypoxia inducible factor*) has also been strongly put forward.[17] The lack of a significant decrease in hepcidin mRNA levels in our TM patients could therefore appear unexpected, since the TM phenotype is known to induce more severe anemia and iron overload than TI. Furthermore, Kattamis *et al.*[9] found the ratio between urinary hepcidin and ferritin was significantly decreased in TM patients compared with controls and hepcidin mRNA expression of these TM patients was found to correlate positively with hemoglobin values. However, these discrepancies could be because: (i) the distinction between TI and TM remains difficult to define;[1] (ii) the therapeutic schedule (chelation-transfusion) is not fully standardized, and may generate variable modifications in iron burden between different sets of patients; (iii) our patients were older than those of Kattamis *et al.*[9] In a report by Kearney *et al.*,[7] the lower urinary hepcidin levels in TI compared with TM were explained by higher ferritin values in TM versus TI. However, as mentioned previously, this was not observed in our series (Table 1).

Differences in hepcidin mRNA levels between TI and TM groups were not related to changes in inflammatory status since there was no difference in CP and CRP levels between the two groups (Table 1). In addition, the slight increase of CRP mRNA levels found in controls was not associated either with an increase in the mRNA levels coding CP, another acute phase protein, suggesting that there was no significant difference in inflammatory status between the three groups, or with a change in hepcidin levels in TM. Altogether, these observations reinforce the hypothesis that the erythropoietic drive plays an important role in hepcidin mRNA expression in thalassemia, regardless of the confounding factors, including the extent of iron overload and inflammation. However, the possible role of splenectomy, which was performed in all thalassemic patients, remains open to debate.

Expression of other iron metabolism genes during thalassemia

Correlations between HJV or TfR2 and hepcidin expression levels have already been found in different types of iron overload.[10,18] HFE, TfR2 and HJV, mutated in different forms of hemochromatosis with hepcidin defect,[13] are thought to play a regulatory role on hepcidin expression. In this study, we found no correlation between these genes and hepcidin mRNA levels (Table 2) suggesting that these mRNA levels probably have no major impact on hepcidin expression during this disease. Furthermore, ferroportin, HFE, hemojuvelin and TfR2 mRNA levels were well correlated in the control group, except for TfR2 versus ferroportin (Table 2). These correlations were maintained in thalassemic groups (mixed or individualized) (Table 2) supporting the theory of an independent regulation of these genes in β-thalassemia disorders. However, we cannot exclude the possible effect of a putative translational regulation on the expression level of these genes, especially for the TfR2 gene product which has been reported to be stabilized by holotransferrin.[19] Nevertheless, we found a slight increase of HFE mRNA expression in TI compared with controls (Table 1). This was also reported in a study using a mouse model of thalassemia.[10] However, this was confirmed for the TM (C57Bl/6 $Hbb^{th3/th3}$) but not for the TI

Table 2. Relationship between hepatic mRNA levels of the different studied genes in the different patient groups.

	Hepcidin	*HFE*	*Hemojuvelin*	*Transferrin receptor 2*
HFE	NS			
Hemojuvelin	NS	*Rho=0.695 p<0.002* Rho=0.817 *p*<0.03		
Transferrin receptor 2	NS	*Rho=0.777 p<0.0004* Rho=0.962 *p*<0.007	*Rho=0.81 p<0.0001* Rho=0.703 *p*<0.05	
Ferroportin	NS	*Rho=0.789 p<0.0003* Rho=0.703 *p*<0.05	*Rho=0.612 p<0.005* Rho=0.711 *p*<0.05	*Rho=0.82 p<0.0001* NS
Ceruloplasmin	NS	ND	ND	ND
C-reactive protein	NS	ND	ND	ND

Hepatic mRNA expression levels of hepcidin, ferroportin, HFE, hemojuvelin, TFR2, ceruloplasmin and C-reactive protein genes were determined by quantitative RT-PCR in the different patient groups. Correlation values were calculated by using the non-parametric Spearman test. Rho and p values were calculated for the thalassemic patients (italic) and control patients (normal). NS indicates that there is no significantly difference between the compared conditions. ND indicates that correlation was not calculated.

($Hbb^{th3/+}$) mice. Furthermore, there was a significant decrease in TfR2 mRNA expression in the liver of these TM mice, which was not observed in our study (Table 1). These differences between mouse beta-thalassemic models and human patients could be related to species differences or to the role of environmental factors. There has been no previous report describing a decrease in hepatic ferroportin mRNA level in TM patients. In our study, this decrease was confirmed (Table 1). In addition, no relationship between ferroportin mRNA expression and hemoglobin levels in thalassemia was seen (*data not shown*). An increase in duodenal ferroportin mRNA levels was reported by Gardenghi *et al.*[15] in a thalassemia mouse model. However, other experiments in mice, with manipulated erythropoietic activity, did not show any change in hepatic ferroportin mRNA.[11] So far, these different findings have made it difficult to identify any possible involvement of ferroportin in the phenotypic expression of thalassemia. In addition, we cannot exclude the possibility of a ferroportin translational regulatory mechanism related to the IRE/IRP system which has been previously reported in other conditions.[20] In conclusion, our study supports the views that the erythropoietic drive is stronger than iron overload for regulating hepcidin expression and does not seem to act through a regulation of HFE, HJV or TfR2 gene expression, and that the regulation of the non-hepcidin iron related genes is relatively independent of the underlying thalassemic disease.

Authorship and Disclosures

EC: conception and design, acquisition of data, analysis and interpretation, drafting article; GZ: conception and design, acquisition of data, analysis and interpretation, text revision; LD; conception and design, analysis and interpretation, text revision; ARL, FS, SV: acquisition of data, analysis and interpretation; M-BT: analysis and interpretation, text revision; EA: acquisition of data, analysis and interpretation, text revision; EAb; analysis and interpretation, text revision; OL: conception and design, analysis and interpretation, text revision; PC: conception and design, analysis and interpretation, text revision; MEL: conception and design, analysis and interpretation, text revision; PB: conception and design, analysis and interpretation, text revision.

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