Effect of α thalassaemia trait and enhanced γ chain production on disease severity in β thalassaemia major and intermedia

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Abstract

One hundred and twenty patients with homozygous β thalassaemia were selected to determine the clinical effects of certain genetic factors which may modify disease severity. Genetic analysis defined specific β thalassaemia mutations, the α thalassaemia genotype, and the presence of an XmnI restriction enzyme site, associated with increased fetal haemoglobin (HbF) production under certain conditions. Genotypic data with globin chain synthesis were related to the age when regular transfusions began and subsequent pubertal development. This study showed that the major determinants of disease severity in β thalassaemia were the β thalassaemia mutations, with co-inheritance of α thalassaemia trait and co-inheritance of a high HbF determinant acting as ameliorating factors. The presence of an α thalassaemia deletion significantly reduced initial disease severity, although the effect on pubertal development was less clear. It is concluded that detailed genetic analysis should be performed in all newly diagnosed patients with thalassaemia. This, in conjunction with clinical assessment, will help to predict disease severity and prognosis.

(Arch Dis Child 1994; 70: 30-34)

Homozygous β thalassaemia (implying defective β globin production from both alleles) presents a spectrum of clinical features. In the severest form the infant needs regular blood transfusions starting from the age of 3-6 months because of life threatening anaemia. Milder cases may present later in childhood with less severe anaemia and deciding whether or not to begin blood transfusions can be difficult. One explanation for the variability in disease severity is the different genetic defects underlying the reduced production of β globin chains. These defects vary from those completely abolishing β globin chain production (β^0) to those allowing some, albeit severely reduced, synthesis (β^+), or mildly reduced synthesis (β^{++}). Patients with homozygous β^0 thalassaemia are usually more severely affected than those with homozygous β^+ or β^{++} defects,¹ but some of the β^+ defects are no less severe than β^0 homozygotes. On the other hand, some forms of heterozygous β thalassaemia are dominant

and result in the clinical picture of thalassaemia intermedia.² The number of active α genes is also known to affect the severity of β thalassaemia, as patients with an excess of α genes (five or six) have a greater degree of α : β chain imbalance and more severe disease than those with a normal number (four).³ Patients with coexisting α thalassaemia (α gene deletion or dysfunction) may, however, have milder disease.⁴ The ability of the patient to synthesise fetal haemoglobin (HbF) may also affect the clinical outcome; for example, in certain patients with $\delta\beta$ thalassaemia or hereditary persistence of HbF, its production is retained at supranormal levels into childhood and adult life.

Other genetic factors, such as a single base substitution C-T at position -158 upstream of the γ gene, are associated with increased HbF production and this is more marked under conditions of erythropoietic stress. This particular mutation can be detected by the presence (+) or absence (-) of an XmnI restriction enzyme site. The association has been studied in sickle cell syndrome,⁵ in β thalassaemia intermedia with certain haplotypes,⁶ in β thalassaemia/HbE disease,⁷ and in homozygous β thalassaemia. The ameliorating effect of the XmnI(+) allele is apparent from these studies. It is not certain, however, whether the XmnI(+) allele acts as an unusual γ chain promoter, or whether it increases γ chain production under erythropoietic stress that codes in the β gene cluster.

In this study we have analysed, in 120 patients with homozygous β thalassaemia, the α and β globin genes, the Xmn allele and α , β , and γ globin chain synthesis in the reticulocyte preparations of 11 patients.

The results of the genotypic analysis and the presence of the XmnI(+) allele have been related to the age of onset of transfusion in 108 patients and the subsequent sexual development in 104 patients. The results show that genotypic features in the α and β globin loci and the XmnI allele can be useful in predicting the subsequent clinical course of patients with homozygous β thalassaemia.

Patients and methods

PATIENTS

One hundred and twenty patients aged 16-26 (mean 21.5) years were studied. Men and

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Accepted 19 August 1993

women were equally represented, with two thirds of patients being of Mediterranean origin and the remaining third of Asian, Indian, Arabic, and Chinese extraction. All patients were treated with regular transfusions of 3–4 units monthly, aiming for an overall mean haemoglobin of 120 g/l. Patients developing hypersplenism of a significant degree (a need for packed red blood cell transfusion >250 ml/kg/year) underwent splenectomy, with a subsequent decrease in transfusion requirement to around 150–180 ml/kg/year. From 1976–7, patients received subcutaneous desferrioxamine infusions in doses of 20–60 mg/kg five to six times a week. In addition, intravenous desferrioxamine (1–2 g/unit of blood) was given at the time of blood transfusions. For children born after 1977, chelation treatment started on average after the twelfth transfusion. The desferrioxamine dose was carefully calculated to avoid problems with growth, vision, and hearing.⁸ We attempted to keep serum ferritin between 1000 and 2000 μ g/l in all patients. Prepubertal and postpubertal serum ferritin concentrations were then considered for each patient.

Non-compliant patients (using subcutaneous desferrioxamine less than five times a week on average) with serum ferritin values >2000 μ g/l prepubertally, or >4000 μ g/l postpubertally were excluded from the analysis of the relation with sexual development. In this way we aimed to minimise differences between the groups for this comparison, which would simply reflect variations in iron overload due to differences in compliance with chelation treatment. Taking this into consideration genotypes were studied in 120 patients; information about disease severity was available in 108 patients and sexual development was studied in 104 patients.

METHODS

DNA was prepared from the buffy coat cells of potassium EDTA anticoagulated blood samples by phenol extraction.⁹ The β thalassaemia mutations were characterised by a polymerase chain reaction based on allele specific amplification (the amplification refractory mutation system, or ARMS).¹⁰ The α thalassaemia deletion genes were characterised by a Southern blot analysis of BamHI digested DNA hybridised to an α gene probe and BglII digested DNA hybridised to a ζ gene probe.¹¹ In addition, the DNA samples were digested with Xmn-I and hybridised to a γ globin gene probe. The mutation (C-T) at position -158 to the G γ gene was detected by amplification across the site using the polymerase chain reaction and analysis by electrophoresis of the digested product.¹² For globin chain synthesis analysis [³H]leucine incubation was performed on samples of heparinised blood taken immediately before blood transfusion. Total counts and specific activity ratios were calculated to give values for α : β , α :non- α , and α : γ ratios.¹³

Definition of disease severity

We defined severe β thalassaemia as that requiring transfusion before the age of 2 years. Our current practice is to start regular transfusion when the haemoglobin level decreases to less than 70 g/l and remains there for a week or more in the absence of other factors such as infection. Transfusion is also considered, even when the haemoglobin is greater than 70 g/l, if there is impaired growth, severe bone change, or hypersplenism.¹⁴ The clinical situation, however, may not be well defined and the haemoglobin level may be less than 70 g/l in a clinically well child who is growing and developing satisfactorily. In this instance, the diagnosis of thalassaemia intermedia should be considered. Because of this large degree of phenotypic heterogeneity we have divided our patients into three clinical groups: (a) severe illness, requiring regular blood transfusions for survival before 2 years of age; (b) late onset, transfusion dependent after 2 years of age; and (c) intermedia, not requiring regular transfusion.

Puberty and sexual development

Pubertal development was studied in all patients with clinical staging as described by Tanner *et al.*¹⁵ The patients were divided into those with normal pubertal development and those with delayed puberty, defined as the absence of pubertal signs in girls over the age of 13 years and in boys over the age of 14 years.

Statistical analysis was by the SPSS computer software package.^{16 17}

Results

Table 1 divides the patients according to ethnic origin and β globin genotype. The known association of each of these genotypes

Table 1 β Thalassaemia mutations (120 patients)

Origin of patients	No of patients	Type of thalassaemia*	β Globin alleles
Mediterranean (n=84)	2	β^0/β^0	IVSI-1/IVSI-1
	4		c39/c39
	1	β^0/β^+	c39/IVSII-745
	3		c39/IVSI-110
	8		IVSI/IVSI-110
	45	β^+/ β^+	IVSI-110/IVSI-110
	1		IVSII-745/IVSII-745
	5		IVSI-110/IVSII-745
	2	β^0/β^{++}	IVSI-1/-101
	2		IVSI-1/-87
	8	β^+/ β^{++}	IVSI-110/IVSI-6
	1		IVSII-745/IVSI-6
Asian/Indian (n=28)	1	β^0/β^0	Fr41-42/Fr41-42
	3		619bpdel/619bpdel
	3		Fr8-9/Fr8-9
	1		Fr41-42/C15
	1		Fr8-9/Fr41-42
	4	β^0/β^+	Fr8-9/IVSI-5
	2		c15/IVSI-5
	1		Fr41-42/IVSI-5
	1		619bpdel/IVSI-5
	4	β^+/ β^+	IVSI-5/IVSI-5
Arabic/Chinese (n=8)	5		IVSI-5/c30(G-C)
	2	β^0/β^{++}	Fr41-42/-28
	2		Fr41-42/CAP+1
	1	β^0/β^0	-25bpdel/c39
	1	β^0/β^+	IVSII-1/IVSI-110
	3	β^+/ β^+	IVSI-5/IVSI-5
	2	β^0/β^{++}	FR41-42/-28
	1	β^+/ β^{++}	IVSI-110/-88

*Thalassaemia associated with mild defects is marked β^{++} . These are mutations -101(C-T), -87(C-G), IVSI-6(T-C), Cap+1(A-C), -88(C-T).

Table 2 Relation of clinical characteristics to genotype and to globin chain synthesis in 11 patients

Clinical details of patients	β Globin genotype	α Globin genotype	Ratio $\alpha:\beta$	Ratio $\alpha:\text{non-}\alpha$	Ratio $\alpha:\gamma$
Severe (n=1); delayed puberty; splenectomy	β^0/β^0	$\alpha\alpha/\alpha\alpha$	No β chain		2:3:1
Severe (n=2); delayed puberty; splenectomy; diabetes mellitus	β^+/ β^+	$\alpha\alpha/\alpha\alpha$	26:1	2:8:1	3:1
Late onset (n=3); normal puberty; splenectomy; one spontaneous and one induced pregnancy	β^+/ β^+	$-\alpha^{3.7}/\alpha\alpha$	5:1		
Late onset (n=4); normal puberty; splenectomy	β^+/ β^+	$-\alpha^{3.7}/\alpha\alpha$	8:2:1	2:9:1	4:4:1
Late onset (n=5); delayed puberty; splenectomy	β^+/ β^+	$-\alpha^{3.7}/\alpha\alpha$	5:3:1	4:4:1	27:1
Late onset (n=6); normal puberty; splenectomy; fathered five children	β^+/ β^{++}	$\alpha\alpha/\alpha\alpha$	4:2:1	1:5:1	2:4:1
Late onset (n=7); normal puberty; two spontaneous pregnancies	β^+/ β^{++}	$-\alpha^{3.7}/\alpha\alpha$	4:1	2:8:1	9:2:1
Intermedia (n=8); normal puberty; splenectomy; one spontaneous pregnancy	β^+/ β^{++}	$-\alpha^{3.7}/\alpha\alpha$	4:1	3:1	4:6:1
Late onset (n=9); normal puberty; splenectomy	β^0/β^+	$\alpha\alpha/\alpha\alpha$	48:1	4:4:1	4:9:1
Late onset (n=10); normal puberty; splenectomy	β^0/β^+	$-\alpha^{3.7}/\alpha\alpha$	No β chain		2:1
Late onset (n=11); normal puberty; splenectomy	β^0/β^+	$-\alpha^{3.7}/\alpha\alpha$	18:1	2:1:1	2:4:1

with β^0 , β^+ , and β^{++} β globin expression is shown in the middle column.

α THALASSAEMIA MUTATIONS

Southern blot analysis showed a high incidence of co-inheritance of α thalassaemia trait. In patients of broadly Mediterranean origin the prevalence of α thalassaemia trait was 27% (23/84 patients), whereas the Asian, Indian, Arabic, and Chinese patients showed a combined prevalence of 17% (6/36 patients). Four patients were carriers for two α globin gene deletions ($--\text{Med}/\alpha\alpha$). All the others were carriers for α^+ thalassaemia with the genotype $-\alpha^{3.7}/\alpha\alpha$. Non-deletional defects affecting the α_2 globin gene were not investigated.

Table 3 Disease severity of 108 patients

β Globin genotype	β Globin alleles	α Globin genotype	Severe disease	Late onset	Intermedia
β^0/β^0	Fr41-42/C15 IVSI-1/TVSI-1 C39/C39 Fr8-9/Fr41-42 Fr41-42/Fr41-42 619bpdel/619bpdel IVSII-654/IVSII-654	$\alpha\alpha/\alpha\alpha$	10	0	0
β^0/β^0	Fr8-9/Fr8-9 619bpdel/619bpdel	$-\alpha^{3.7}/\alpha\alpha$	4	0	0
β^0/β^+	Fr41-42/CAP+1 C39/IVSI-110 Fr8-9/IVSI-5 IVSI-1/IVSI-110 Fr8-9/IVSI-5 FR41-42/-28 C39/IVSII-745 Fr41-42/IVSI-5 C15/IVSI-5	$\alpha\alpha/\alpha\alpha$	18	0	0
β^0/β^+	IVSI-1/IVSI-110 C15/IVSI-5	$-\alpha^{3.7}/\alpha\alpha$	8	1	0
β^0/β^{++}	IVSI-1/-87 IVSI-1/-101 Fr41-42/CAP+1	$\alpha\alpha/\alpha\alpha$	4	0	0
β^+/ β^+	IVSI-110/IVSI-110 IVSI-110/IVSII-745 IVSI-5/IVSI-5 IVSI-110/IVSII-745	$\alpha\alpha/\alpha\alpha$	40	0	0
β^+/ β^+	IVSI-110/IVSI-110 IVSI-5/c30 G→C	$-\alpha^{3.7}/\alpha\alpha$	3	10	0
β^+/ β^{++}	IVSI-110/IVSI-6 IVSII-745/IVSI-6 IVSI-110/-88	$\alpha\alpha/\alpha\alpha$	0	5	0
β^+/ β^{++}	IVSI-110/IVSI-6	$-\alpha^{3.7}/\alpha\alpha$ $--\text{Med}/\alpha\alpha$	0	2	3

Xmn-I RESTRICTION ENZYME SITE

Patients were also characterised according to whether they were homozygous or heterozygous for the presence (+) of an XmnI restriction enzyme site. One patient of Arabic origin was found to be homozygous (+/+), whereas 2% (2/84 patients) of the Mediterranean group and 30% (11/36 patients) of the Asian, Indian, Arabic, and Chinese group were heterozygous (-/+).

Table 2 shows the relation of clinical characteristics to genotype and to the amount of globin chain synthesis in 11 patients. None or a very small amount of β globin chain is produced in β^0/β^0 or β^+/ β^+ genotypes with full complements of α genes. In this group the disease was severe and pubertal development delayed. Less α chain excess is present in patients with the β^+/ β^+ , β^+/ β^{++} genotype with α gene deletion and these patients started transfusion later in life and had normal pubertal development. Patients with β^+/ β^{++} and α gene deletion with $\alpha:\beta$ globin chain ratio 4:1 are the intermediate types, and required only occasional transfusions with normal pubertal development and normal reproductive capabilities.

DISEASE SEVERITY

Patients with the β^0/β^0 genotype all started regular transfusion within the first two years of life irrespective of a co-inheritance of α thalassaemia (table 3). A significant difference was seen in the β^+/ β^+ group where all 40 patients with four α genes had severe disease but most with an α thalassaemia deletion were in the late onset group. This effect was tested using χ^2 analysis with Yates's correction, yielding a result of 29.68 with one degree of freedom ($p < 0.001$; ϕ coefficient 0.28). Also one of nine patients in the β^0/β^+ group with α thalassaemia trait was in the late onset group. None of the patients with the β^+/ β^{++} genotype were severe and three of five with α thalassaemia were intermedia (table 3).

PUBERTAL DEVELOPMENT

Patients were divided into those achieving normal puberty and those with delayed puberty (table 4). Prepubertal and postpubertal serum ferritin values showed no significant differences between patients with the different β thalassaemia alleles as shown with the Kruskal-Wallis test value of 0.5 with two degrees of freedom ($p > 0.05$). In the β^0/β^0 , β^0/β^+ , and β^+/ β^+ groups, normal puberty was achieved in almost one third of patients. Patients with β^+/ β^+ or β^+/ β^{++} genotypes and coexisting α thalassaemia trait were more likely to enter normal puberty than those with less favourable genotypes (13/18; 72%). The numbers were not large enough to achieve statistical significance at the 5% level, however.

XmnI RESTRICTION SITE

For the XmnI restriction enzyme site, one patient with the XmnI(+/-) genotype with

Table 4 Correlation of genotype with pubertal development in 104 patients

β Globin genotype	α Globin genotype	No with normal puberty	No with delayed puberty
β^0/β^0	$\alpha\alpha/\alpha\alpha$	4	6
	$-\alpha^{3.7}/\alpha\alpha$	1	3
β^0/β^+	$\alpha\alpha/\alpha\alpha$	6	12
	$-\alpha^{3.7}/\alpha\alpha$	2	7
β^+/ β^+	$\alpha\alpha/\alpha\alpha$	10	30
	$-\alpha^{3.7}/\alpha\alpha$	8	5
β^+/β^{++}	$\alpha\alpha/\alpha\alpha$	5	
	$-\alpha^{3.7}/\alpha\alpha$	5	

β^0/β^0 thalassaemia and a complete complement of α genes was thalassaemia intermedia in type. The other 13 patients with XmnI(-/+) genotypes were late onset phenotypes irrespective of their β or α genotype.

Discussion

The results of this study add further evidence for establishing the molecular basis of the child with thalassaemia as an aid to the prediction of disease severity (thalassaemia major or intermedia). The main determinant is the inheritance in a homozygote or compound heterozygote fashion of mutations altering β globin chain production from the affected β globin loci. In our Mediterranean patients, known 'mild' thalassaemia genes, causing less severe depression of the β chain synthesis were the IVSI-6 and IVSI-87 mutations. The IVSI-6 or Portuguese type of mutation was first described by Orkin *et al* and Tamagnini *et al*.¹⁹ It is a single base substitution T-C and in the homozygous state is the most common cause of thalassaemia intermedia in many Mediterranean countries. Homozygous patients have a mild anaemia, normal, or slightly increased HbF and are not transfusion dependent. Ten of our Mediterranean patients were compound heterozygotes for this mutation and they all showed a milder form of the disease with late onset of transfusions and normal sexual development.

The IVSI-87 promoter mutation is also a known mild β thalassaemia allele, able to produce a phenotype of intermediate severity even in combination with β^0 thalassaemia. Our patient with this mutation is in the late onset group. Rosatelli *et al* reported clinical data for nine patients doubly heterozygous for codon 39 nonsense mutation (β^0) and the IVSI-87 promoter mutation.²⁰ Four patients were not transfused, two sporadically, and three regularly transfused.

A C-T substitution at nt-101 in a conserved DNA sequence of the promoter region of the β globin gene is associated with silent β thalassaemia.²¹ Heterozygotes have normal concentrations of HbA₂ and a mild imbalance in chain synthesis. Our patient compound heterozygous β^0/β^{++} with C-T substitution at nt-101 started transfusions at the age of 4 years.

We have two patients, one of Indian origin the other of Arabic extraction, with Cap-1 and Cap-88 mutations. The Indian patient homozygous for Cap-1 started transfusions at the age of 4 years whereas the compound heterozygote IVSI-101/-88 Arabic patient

started transfusions at the age of 32 years.

These data show that the co-inheritance of α thalassaemia, especially in the form of two α globin gene deletions (non-deletional defects were not tested for in this study) may be associated with β^+/ β^+ or β^+/β^{++} resulting in thalassaemia intermedia. This ameliorating effect is best illustrated in one of our families of three siblings aged 43, 39, and 40 years.

All three inherited a β^+/β^{++} mutation. The 43 year old subject has normal α genes and has received regular transfusions since the age of 10 years. The 39 and 40 year old subjects co-inherited α thalassaemia trait. They are sporadically transfused. All three are married and have nine children between them.

The presence of an XmnI site 5' to the Gy gene at position -158 has been associated with increased expression of the Gy gene in sickle cell anaemia, β thalassaemia intermedia, and β thalassaemia haemoglobin E disease. Thein *et al* have shown that XmnI(+/+) subjects have marginally increased concentrations of haemoglobin F compared with the XmnI(-/-) subjects.⁶ Our data show that the XmnI allele is much less common in Mediterranean (Greek, Italian, Cypriot) patients than in patients of Chinese, Asian, India, or Arabic origin. We had only one patient with the XmnI(+/+) mutation associated with β^0 thalassaemia. She is β^0/β^0 with complete α genes and, at the age of 14 years, does not require transfusions. All 13 patients with XmnI(-/+) showed a late onset of thalassaemia major independently of the severity of the co-inherited β mutation, although none were β^0/β^0 .

The results of this study are consistent with the view that the clinical spectrum of β thalassaemia can be largely explained by the following factors: the inheritance of severe or mild β thalassaemia mutations (the major determinant), the co-inheritance of α thalassaemia, and the co-inheritance of a high HbF determinant. Genotypic data help to predict disease severity and assist judgement about the prognosis of individual patients.

We thank Professor Sir David Weatherall FRS for his helpful advice and constructive criticism and Andrea Wonke for typing the manuscript.

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