Molecular analysis of $\beta$-thalassaemia patients in a high incidence area of southern Italy

L. RIGOLI*, A. MEO†, M.R. MICELI†, K. ALESSIO†, R.A. CARUSO‡, M.A. LA ROSA‡, D.C. SALPIETRO*, M. RICCA‡, I. BARBERI*

*Department of Paediatrics, University of Messina, School of Medicine, Italy, †Paediatric Department of Thalassaemia Ward, University of Messina, School of Medicine, Italy and ‡Department of Human Pathology, University of Messina, School of Medicine, Italy

Summary
The prevalence of eight mutations in 84 patients with $\beta$-thalassaemia major and in 16 subjects with thalassaemia intermedia was investigated. All of the patients were Italian, originating from Eastern Sicily (Messina area) and some Calabrian regions. Genomic DNA was amplified by polymerase chain reaction (PCR). DNA molecular investigations were performed by allele-specific oligonucleotide (ASO) hybridization, to identify the following $\beta$-thalassaemia mutations: CD39 (C-T), IVS1-110 (G-A), IVS1-6 (T-C), IVS1-1 (G-A), IVS2-745 (C-G), IVS2-1 (G-A), −87 (C-G), CD6 A (−A).

Our data underline that in thalassemia intermedia two mutations were statistically prevalent: IVS1-6 $\rightarrow$ C ($P < 0.001$) and CD 6-A ($P < 0.05$). CD 39 was statistically prevalent in $\beta$-thalassaemia major patients ($P < 0.01$). The difference between the two groups was not statistically significant for all the other mutations. Five different genotypes were recorded among thalassaemia intermedia and 15 among $\beta$-thalassaemia major patients. Twenty-five percent of the intermedia patients and 4.5% of the major patients had homozygosity for mild mutations (group I); 62.5% of the intermedia patients and 26.2% of the major patients had combinations of mild/severe mutations (group II). In addition, homozygosity or double heterozygosity for severe mutations (group III) was found in 12.5% of the intermedia patients and 69% of the major patients. Some genotypes were restricted to thalassaemia intermedia, including heterozygosity −87/IVS1-6 and IVS1-6/CD 6-A. It is essential to understand the distribution and frequency of the relevant mutations in each population where $\beta$-thalassaemias exist. This is of particular importance for genotype–phenotype correlation and for carrier detection, genetic counselling and prenatal diagnosis.

Keywords $\beta$-thalassaemia, mutations, PCR, ASO

Introduction
The $\beta$-thalassaemias are a heterogeneous group of inherited disorders caused by mutations in and around the structural gene of the adult haemoglobin (HbA) $\beta$-chain (Weatherall & Clegg, 1981; Orkin & Kazazian, 1984). Studies at the gene level have identified a large number of $\beta$-thalassaemia gene variations in different populations (El-Hazmi et al., 1983; Wong et al., 1992). The clinical severity of $\beta$-thalassaemia can be influenced by genetic determinants that are capable of reducing globin chain imbalance. Clinically, $\beta$-thalassaemias may be mild or silent conditions, or they may cause a severe disease, leading to early transfusion dependence (Gelehrter & Collins, 1990). In the latter case, affected individuals
present with severe anaemia in early infancy, requiring lifelong transfusion and iron chelation therapy (β-thalassaemia major) (Orkin & Kazazian, 1984). In a small minority of cases, the inheritance of two β-thalassaemia alleles results in a disease of moderate severity known as β-thalassaemia intermedia. β-thalassaemia intermedia may result from the inheritance of two β+-thalassaemia alleles or the combination of a severe β+-thalassaemia allele and a particularly mild or silent β+-thalassaemia allele (Camaschella & Cappellini, 1995; Camaschella et al., 1959). Sophisticated molecular techniques are required for genotypic identification. In this study, DNA molecular investigations were performed along with conventional laboratory investigations in a group of major and intermedia β-thalassaemia patients.

Patients and methods

The study was approved by the Committee for Ethics in Medical Research and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. All subjects gave their informed consent prior to their inclusion in the study.

The study population included 84 patients with β-thalassaemia major and 16 subjects with thalassaemia intermedia. All of the patients were Italian, originating from eastern Sicily (Messina area) and some Calabrian regions, and attended our Paediatric Department Thalassaemia Ward. There were no interrelationships among the patients. β-thalassaemia characterization studies included family history, clinical and haematological investigations and biosynthesis studies. All of the thalassaemia major patients were on a regular transfusion regimen. Four of the 16 thalassaemia intermedia patients (25%) had never received blood transfusions. Ten patients (62.5%) had received occasional transfusions, two patients (12.5%) had received transfusions as adults.

The mean age of the β-thalassaemia major patients was 13 years (range 5–18 years), while the mean age of the thalassaemia intermedia group was 27 years (range 11–53 years).

Blood samples for routine haematological analysis were drawn in EDTA tubes. For haemoglobin phenotyping, a sample of whole blood was analysed by electrophoresis at alkaline pH. HbA2 (Marengo Rowe, 1965) and Hbf (Betke et al., 1959) were estimated and the α/β chain ratio was determined (El).

Genomic DNA was extracted from blood samples (Sambrook, 1989) and amplified by the polymerase chain reaction (PCR) (Saiki et al., 1988; Cai et al., 1989). Allele-specific oligonucleotide (ASO) hybridization (Old et al., 1990) was used to determine the following β-thalassaemia mutations: CD39 (C-T), IVS1-110 (G-A), IVS1-6 (T-C), IVS1-1 (G-A), IVS2-745 (C-G), IVS2-1 (G-A), -87 (C-G), CD6-D (Table 1). The β-thalassaemia mutations screened were those commonly encountered in the Mediterranean population (Di Marzo et al., 1990; Kattamis et al., 1990; Murru et al., 1991). Oligonucleotide primers used for ASO hybridization were purchased commercially. PCR was carried out in a 2400 Perkin–Elmer Thermocycler. A denaturation step at 94 °C for 1 min was followed by 34 cycles of denaturation (94 °C, 45 s), annealing (62 °C, 15 s), and primer extension (72 °C, 30 s). The final extension was performed at 72 °C for 7 min. The PCR products were separated by electrophoresis on a 2.0% agarose gel and visualized by ethidium bromide staining and ultraviolet light illumination prior to ASO hybridization.

Results

Different gene mutations were detected in the two groups of patients (β-thalassaemia major and intermedia), and at different frequencies.

In the β-thalassaemia major patients the following mutations were detected: IVS1-110 (34.5%), CD39 (34.5%), IVS1-1 (10.7%), IVS1-6 (9.5%), IVS1-745 (6.0%), IVS1-1 (2.4%), -87 (2.4%) (Table 2). Several patients (16/84) were homozygous for the IVS1-110

<table>
<thead>
<tr>
<th>Location</th>
<th>Mutation</th>
<th>Effect</th>
<th>β-thalassaemia Type</th>
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<tbody>
<tr>
<td>IVS1-110</td>
<td>G→A</td>
<td>Internal IVS change a</td>
<td>β+</td>
</tr>
<tr>
<td>CD 39</td>
<td>C→T</td>
<td>Nonsense mutant b</td>
<td>β°</td>
</tr>
<tr>
<td>IVS1-1</td>
<td>G→T</td>
<td>Splice junction change a</td>
<td>β°</td>
</tr>
<tr>
<td>CD 6 A</td>
<td>A→T</td>
<td>Frameshift mutant b</td>
<td>β°</td>
</tr>
<tr>
<td>IVS1-1</td>
<td>G→A</td>
<td>Splice junction change a</td>
<td>β°</td>
</tr>
<tr>
<td>IVS1-6</td>
<td>T→C</td>
<td>Consensus change a</td>
<td>β+</td>
</tr>
<tr>
<td>IVS1-745</td>
<td>C→G</td>
<td>Internal IVS changes</td>
<td>β+</td>
</tr>
<tr>
<td>-87</td>
<td>C→G</td>
<td>Transcriptional mutant</td>
<td>β+</td>
</tr>
</tbody>
</table>

Table 1. β-thalassaemia mutations tested in Eastern Sicily and Calabria regions

a. RNA processing mutant; b. nonfunctional RNA.
mutation. In this group, the mean ferritin level was 1500 ng/ml, the mean age of first transfusion was 14 months, and the mean transfusion requirement was 17 per year.

CD 39 homozygosity was seen in 16 patients. In this group, the mean ferritin level was 2750 ng/ml, the mean age of first transfusion was 13 months and the mean transfusion requirement was 18 per year.

IVS1-6 homozygosity was seen in four patients. In this group, the mean ferritin level was 1339 ng/ml, the mean age of first transfusion was four years and the mean transfusion requirement was 14 per year.

IVS1-1 homozygosity was found in four patients. In this group, the mean ferritin level was 800 ng/mg, the mean age of first transfusion was 15 months and the mean transfusion requirement was 16 per year.

Twenty-four patients (28.5%) were IVS1-110 compound heterozygotes for other mutations. Heterozygosity for other mutations was found as follows: CD 39 heterozygosity in 20 patients, IVS1-1 heterozygosity in seven patients. IVS1-745 heterozygosity in five patients, IVS1-6 heterozygosity in four patients, IVS1-1 heterozygosity in three patients and -87 heterozygosity in one patient.

In the thalassaemia intermedia patients, the most frequent mutations were IVS1-6 (50%), IVS1-110 (31.3%), CD 6-A (12.5%) and -87 (6.2%) (Table 3).

Three of the patients (18.8%) were homozygous for the IVS1-110 mutation. In this group, the mean ferritin level was 1018 ng/ml, the mean age of first transfusion was six years and the mean transfusion requirement was 22 per year.

In four patients with IVS1-6 homozygosity the mean ferritin level was 354 ng/mg, the mean age of first transfusion was 10 years, and the mean transfusion requirement was 11 per year.

In the remaining patients, heterozygosity for other mutations was found as follows. Two patients with -87/

<table>
<thead>
<tr>
<th>Table 2. Frequency of mutations in β-thalassaemia major patients</th>
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<tbody>
<tr>
<td>Mutation location</td>
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<tr>
<td>-------------------</td>
</tr>
<tr>
<td>IVS1-110</td>
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<tr>
<td>CD 39</td>
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<tr>
<td>IVS1-745</td>
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<tr>
<td>IVS1-1</td>
</tr>
<tr>
<td>-87</td>
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<tr>
<td>CD 6-A</td>
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Number of β-thalassaemia major patients = 84.

<table>
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<tr>
<th>Table 3. Frequency of mutations in β-thalassaemia intermedia patients</th>
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<tbody>
<tr>
<td>Mutation location</td>
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<tr>
<td>-------------------</td>
</tr>
<tr>
<td>IVS1-6</td>
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<tr>
<td>IVS1-110</td>
</tr>
<tr>
<td>CD-A</td>
</tr>
<tr>
<td>-87</td>
</tr>
</tbody>
</table>

Number of β-thalassaemia intermedia patients = 16.

IVS1-6 had never received any blood transfusions. In four patients with an IVS1-6/CD 6 genotype, the mean ferritin level was 1100 ng/mg, the mean age of first transfusion was four years and the mean transfusion requirement was 19 per year. In three patients with an IVS1-6/IVS1-110 genotype the mean ferritin level was 1768 ng/ml, the mean age of first transfusion was eight years and the mean transfusion requirement was 10 per year.

The differences in mutation distribution between the major and intermedia groups were assessed statistically. In thalassaemia intermedia, prevalence data for two mutations were statistically significant. These were IVS1-6 T→C $(P < 0.001)$ and CD 6-A $(P < 0.05)$. In the β-thalassaemia major patients, the prevalence of CD 39 was statistically significant $(P < 0.01)$. The differences between the two groups were not statistically significant for any other mutations.

According to these results, the IVS1-6 T→C and -87 C→G mutations may be considered as ‘mild’. Severe mutations included β° CD 39 C→T, which was statistically prevalent in the thalassaemia major group, IVS1-110 G→A and IVS2-745 C→G, which are severe in vitro (Bunn and Forget, 1986) and all the mutations (IVS1-1 G→A, IVS1-1 G→A) which cause β°-thalassaemia (Orkin & Kazazian, 1984).

**Genotypes**

Five different genotypes were recorded among β-thalassaemia intermedia patients and 15 among β-thalassaemia major patients. To simplify genotype-phenotype analysis, the genotypes were classified in three groups, according to mutation severity (see above). Group I included patients with two mild mutations, group II included patients with combinations of mild/severe mutations and group III included patients with two severe mutations (Table 4). Twenty-five percent of the intermedia and 4.8% of the major patients were included in group I; 62.5% of the intermedia and 26.2% of the major patients were included in group II, while group III included 12.5% of the intermedia and 69%
of the major patients (Table 4). Some genotypes were seen only in thalassaemia intermedia patients, including heterozygosity –87/IVS1-6 (two patients in group I) and IVS1-6/CD 6-A (four patients in group II).

### Discussion

The molecular defects in β-thalassaemia are heterogeneous, with over 150 β-thalassaemia mutations reported in the literature (Weatherall & Clegg, 1981; Orkin et al., 1984). Although population studies have revealed a wide spectrum of molecular pathology in β-thalassaemia, limited ethnic group-specific alleles account for over 90% of the defective genes (Tamagnini et al., 1983; Galanello et al., 1986; Wong et al., 1986; Di Marzo et al., 1988; Galanello et al., 1989; Kattamis et al., 1990; Old et al., 1990; Murru et al., 1991; Rosatelli et al., 1992; El-Hazmi et al., 1995). It is therefore important to understand the distribution and frequency of the relevant mutations in each population in which β-thalassaemia occurs.

In the Mediterranean area, β-thalassemias are encountered at a relatively high frequency (Tamagnini et al., 1983; Wong et al., 1986; Bunn et al., 1986; Di Marzo et al., 1988; Galanello et al., 1989; Kattamis et al., 1990; Old et al., 1990; Murru et al., 1991). Genetic counselling of couples at risk for β-thalassaemia is complicated by the variable relationship between genotype and phenotype (Cao et al., 1994). This is particularly true for genotypes involving mild β+-thalassaemia mutations such as IVS1-6 (T-C). For example, the IVS1-6 (T-C)/ codon 39 (C-T) genotype has been reported in both β-thalassaemia major and β-thalassaemia intermedia patients (Waye et al., 1995).

In this report, we have described the range of phenotypes associated with genotypes involving the most common β-thalassaemia mutations in Eastern Sicily (Messina area) and some Calabrian regions. Two mutations were statistically prevalent in thalassaemia intermedia: IVS1:6 T→C (P<0.01) and CD 6:A (P<0.05). CD 39 was statistically prevalent in thalassaemia major (P<0.01).

Classification of our patients into three groups by β-genotype identified four homozygotes for mild mutations (IVS1:6/IVS1:6) in group I who were major patients, receiving their first transfusions at a mean age of 8 months. Two other patients with the same genotype suffered only with thalassaemia intermedia, receiving their first blood transfusions at a mean age of 10 years. According to recent studies (Ragusa et al., 1992; Murru et al., 1992; Efremov et al., 1994; Camaschella et al., 1995), this phenotypic variation cannot be attributed to sequences within the β-globin gene cluster. Moreover, combinations of mild and severe mutations were present both in the intermedia and in the major groups. In our patients, IVS1 : 6 T→C in combination with severe mutations such as CD 39 and IVS1 : 110 was associated with a milder phenotype. It is well known that IVS1 : 6 C→T mutation in vitro creates an alternative splice site which is rarely used, so that normal β chains are produced in significant amounts (Waye et al., 1995). There is general agreement that this mutation is also mild in vivo. Heterozygotes for IVS1 : 6 C→T have less severe red cell changes (Efremov et al., 1994); in the homozygous state, the phenotype is usually that of thalassaemia intermedia, although some patients with thalassaemia major have been reported (Chehab et al., 1989; Meloni et al., 1983). This was also seen in our study.

Our study underlines the relative roles of β+ and β° genes in thalassaemia major homozygotes. It is known that about half the β-thalassaemia alleles lead to complete inactivation of the β-globin gene, causing β°-thalassaemia. The β+-thalassaemia mutations allow some production of β-globin, but the output is reduced. The reduction in β-globin output ranges from minimal to almost complete absence. The severity of these β+ thalassaemia alleles can be correlated to the degree of reduction in MCV in heterozygotes. Heterozygotes for such mutations have normal Hb A2 levels and normal red cell indices and are often referred to as ‘silent’ carriers.

In our study, double heterozygotes for CD39/IVS1-6 or IVS1-110/IVS1-6 could have either intermedia or major disease. Differentiation of Cooley’s disease from thalassaemia intermedia is imprecise. Thalassaemia intermedia is a term applied to a disease characterized by a transfusion-independent clinical course of intermediate severity between thalassaemia major and the asymptomatic carrier state. As the definition of thalassaemia intermedia is relative, it is clinically and genetically

<table>
<thead>
<tr>
<th>Group</th>
<th>Mild/Mild</th>
<th>Mild/Severe</th>
<th>Severe/Severe</th>
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</thead>
<tbody>
<tr>
<td>Thalassaemia major</td>
<td>4/84 (4.8%)</td>
<td>22/84 (26.2%)</td>
<td>58/84 (69%)</td>
</tr>
<tr>
<td>Thalassaemia intermedia</td>
<td>4/16 (25%)</td>
<td>10/16 (62.5%)</td>
<td>2/16 (12.5%)</td>
</tr>
</tbody>
</table>

Table 4. β-genotypes in the major and intermedia groups β-genotypes

heterogeneous. The spectrum includes, at one end, patients with haemoglobin levels of 6 g/dl, skeletal abnormalities and significant transfusion requirements and, at the other end, asymptomatic subjects with mild anaemia and splenomegaly diagnosed by chance or during family studies.

Transfusion independence is an unsatisfactory criterion for defining thalassaemia intermedia, as it depends to a large extent on patient perceptions and medical trends. The introduction of a policy requiring high-level transfusion regimens in infancy for homozygous thalassaemia patients may have led to the enrolment of some patients who would otherwise have been classified as intermedia. On the other hand, as the prognosis of thalassaemia intermedia was considered better than that of patients regularly transfused, some major patients maintaining adequate Hb levels by massive haemopoietic expansion, with associated bone abnormalities, were not enrolled in such programs. New clinical and molecular criteria are clearly required.

Our observations indicate that the clinical phenotype associated with individual \( \beta \)-thalassaemia cases cannot be predicted accurately based only on the \( \beta \)- and \( \alpha \)-globin genotypes. Nevertheless, it is possible that concomitant \( \alpha \)-thalassaemia may ameliorate the clinical severity of \( \beta \)-thalassaemia when other genetic determinants are also present. Such determinants may ameliorate the disease severity by increasing \( \beta \)-globin gene expression or the level of Hb F. It is not easy to predict whether an \( \alpha \)-thalassaemia gene would invariably ameliorate disease severity in homozygous \( \beta^{0} \)-thalassaemia.

Our data underline the importance of molecular investigation to clarify the phenotypic variability of thalassaemias in countries with a high prevalence of haemoglobinopathies. Genotype–phenotype correlation is useful for clarification of the clinical course and will contribute to the eradication of thalassaemias in future generations.

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