

# Association of $\alpha$ globin gene quadruplication and heterozygous $\beta$ thalassemia in patients with thalassemia intermedia

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## ABSTRACT

Ten patients with thalassemia intermedia with variable severity and apparent simple heterozygosity for  $\beta^0$  39 C>T nonsense mutation were submitted to clinical, hematologic and molecular studies. The presence of an unknown molecular defect (silent  $\beta$ -thalassemia) unlinked to the  $\beta$  cluster interacting with the heterozygous  $\beta$  thalassemia, was previously postulated in these families. Analysis of the  $\alpha$  globin gene cluster with PCR-based methods (MLPA, GAP-PCR, digestion with restriction enzymes) detected complex rearrangements in the  $\alpha$  cluster. A duplication of the  $\alpha$  globin gene locus, including the upstream regulatory region, was present in all the patients, associated in some of them with deletion or non-deletion  $\alpha$  thalassemia. The variability of the clinical phenotype correlates with the degree of the globin chain imbalance. The presence of  $\alpha$

globin cluster duplication should be considered in patients heterozygote for  $\beta$ -thalassemia with thalassemia intermedia phenotype and in the carriers of suspected silent  $\beta$  thalassemia.

**Key words:** thalassemia intermedia,  $\alpha$ -globin gene quadruplication, silent  $\beta$  thalassemia, MLPA.

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## Introduction

Variable clinical manifestations are associated with  $\beta$ -thalassemia spanning from the transfusion-dependent condition of thalassemia major to the asymptomatic thalassemia carrier state. Thalassemia intermedia lies between the two extreme phenotypes and comprises a broad spectrum of clinical conditions of different severity having in common the non-transfusion dependence. Part of the clinical heterogeneity of thalassemia intermedia depends on different and only partially defined molecular mechanisms, the most common being homozygosity for mild  $\beta$ -thalassemia mutations, compound heterozygosity for mild/silent and severe  $\beta$ -thalassemia mutations, co-inheritance with homozygous  $\beta$  thalassemia of  $\alpha$  thalassemia or of genetic determinants able to increase the production of fetal hemoglobin (HbF).<sup>1-3</sup> Less commonly, the phenotype of thalassemia intermedia has been reported in subjects carrying only one  $\beta$  globin gene defect. Among them 3 groups have been defined: (i) the dominantly inherited  $\beta$  thalassemia mutations (also reported as inclusion body thalassemias);<sup>4,5</sup> (ii) the co-existence of somatic deletions of a region of chromosome 11 p15;<sup>6,7</sup> (iii) co-inheritance of triplicated  $\alpha$  globin genes with excessive  $\alpha$  globin production.<sup>8</sup> Recently cases of simple  $\beta$  thalassemia heterozygosity presenting with an intermediate to severe phenotype due to duplications of the complete  $\alpha$  globin gene cluster, including the upstream regulatory element HS-40, have been reported;<sup>11</sup> (iv)

interaction between severe and silent  $\beta$  thalassemia mutations.<sup>12,13</sup>

Herein, we report the molecular characterization of 10 individuals belonging to four families, with the clinical phenotype of thalassemia intermedia of variable severity due to the association of  $\alpha$  globin gene quadruplication with heterozygous  $\beta$  thalassemia. These families have been reported before and to explain the thalassemia intermedia phenotype, we postulated the presence of an unknown molecular defect (silent  $\beta$  thalassemia), unlinked to the  $\beta$  globin gene cluster, interacting with the heterozygous  $\beta$  globin gene mutation.<sup>12,13</sup>

## Design and Methods

We studied the clinical and hematologic characteristics and the molecular bases of 10 patients (5 males and 5 females) with  $\beta$  thalassemia intermedia belonging to four Sardinian families (two of them are related). Peripheral blood samples were obtained as part of the clinical study after informed consent.

## Hematologic analysis

Red blood cell indices were determined with Coulter Max -M (I.L. Milan, Italy). Hb A<sub>2</sub> and Hb F quantitation was performed by high performance liquid chromatography, HPLC (Variant, BIO-RAD, Milan Italy). Globin chain synthesis analysis was performed on the peripheral blood reticulocytes following the

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method of Kan *et al.*<sup>14</sup> Appropriate methods have been used to exclude the presence of associated red blood cell membrane defects or enzymatic deficiencies.

### DNA analysis

DNA extraction was performed according to standard methods. Mutation analysis of the  $\beta$  globin gene including the promoter up to 600bp was performed by direct DNA sequencing. The HS2 and HS3 cores have also been sequenced.  $\alpha$ -globin genotype was defined by GAP PCR for the deletional defects and the triple  $\alpha$  gene arrangement, and by digestion of amplification products with NcoI and HphI restriction enzymes for non-deletional defects.<sup>15</sup> To detect the less common anti- $\alpha$  4.2 triplication, the Southern blot analysis was carried out.

To investigate for the presence of extended deletions or duplications on the  $\alpha$  cluster, MLPA method (multiplex ligation-dependent probe amplification) was performed using a set of 25 probes, covering a region of 170 Kb in the  $\alpha$  cluster on the short arm of the chromosome 16 (MRC-Holland, Amsterdam, Nederland, P140B HBA probemix).<sup>16</sup>

## Results and Discussion

Pedigrees of the families, clinical and hematologic data are reported in Figure 1 and Table 1.

All patients with thalassemia intermedia (indicated by the arrows) have moderate to severe microcytic anemia (Hb 7.0-9.8 g/dL, MCV 57-65 fL), increased HbA2 (>4.1%) and HbF (2.7-19.4%) and spleen enlargement. Nine have moderate to marked thalassemia-like bone modifications. Four patients have nucleated red blood cells in their peripheral blood. Serum ferritin and unconjugated bilirubin are normal or moderately increased. Gallstones are present in 4 patients. All patients have normal cardiac function (left ventricle ejection fraction >60%) and pulmonary hypertension

was evident. Among the patients, two different severity degrees can be identified. Patients IV-2 and V-7 in family B-D and patient III-1 in family G have a more severe phenotype (*Table 1 in bold*) [lower hemoglobin levels, marked splenomegaly, severe thalassemia-like bone modifications, need for splenectomy for worsening of anemia in V-7, and needs for repeated red blood cell transfusions from age 4 in III-1 and markedly unbalanced  $\alpha/\beta$  globin chain synthesis ratio ( $\alpha/\beta$  4.0 – 4.2)]. Patients IV-4 and V-4 from family B-D, patients II-1, III-1 and III-2 from family C and patients II-1 and II-2 from family G have a milder phenotype and less unbalanced  $\alpha/\beta$  globin chain synthesis ratio (Table 1). Subject II-2 from family C, despite the presence of  $\alpha$  cluster duplication, has the clinical phenotype of the  $\beta$  thalassemia carrier state: no clinical symptoms, hemoglobin level of 10.5 g/dL, lowest  $\alpha/\beta$  globin synthesis ratio (1.9).

### DNA analysis

Despite the extended analysis of the  $\beta$  globin gene cluster [ $\beta$  globin gene sequence including the polymorphic (AT)<sub>n</sub> (T) sequence at position -530, which has been associated in some studies<sup>17,18</sup> with silent  $\beta$  thalassemia, and sequencing of the HS2 and HS3 core<sup>19,20</sup> of the Locus Control Region], as previously reported,<sup>12,13</sup> all patients have as only abnormality the codon 39 C→T nonsense mutation in one  $\beta$  globin gene and a completely normal sequence in the other. With family studies we easily excluded that the determinant responsible for the severe phenotype resided within the  $\beta$  globin gene cluster because other members of the family heterozygotes for the  $\beta$  39 mutation (I-1 in family C, V-1, V-2, V-5 and V-8 in family B-D, and I-1 in family G) had the typical carrier state phenotype. Moreover, haplotype analysis according to Orkin *et al.*<sup>21</sup> showed the  $\beta$  39 mutation associated with haplotype 1 in B-D family and 2 in C and G families.

$\alpha$  globin gene analysis excluded in all patients with thalassemia intermedia the triple  $\alpha$  globin gene arrangement,

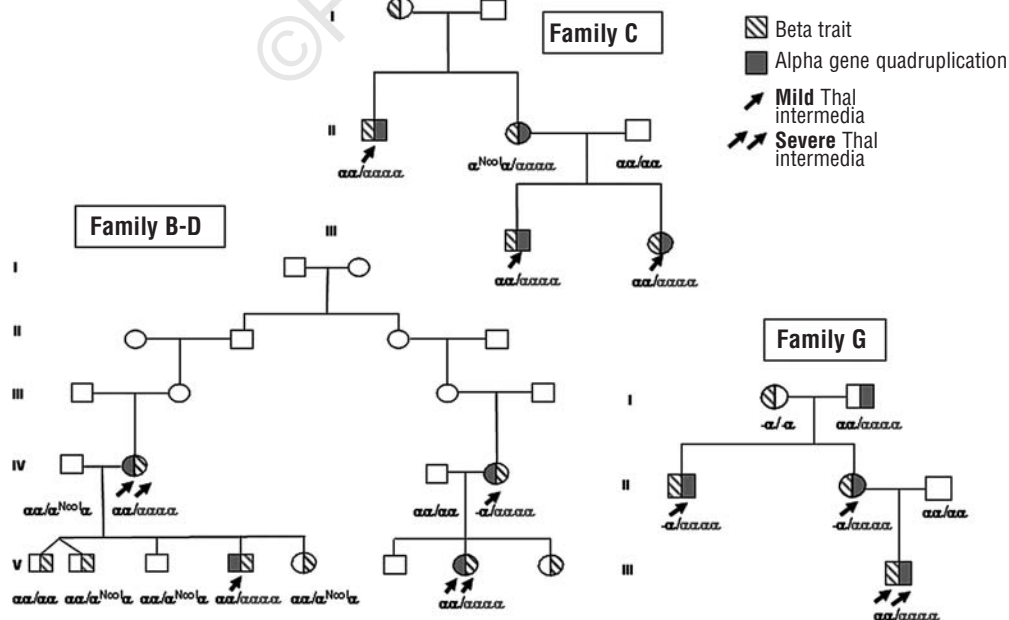


Figure 1. Pedigrees of the families.

**Table 1.** Clinical and hematologic data of the patients (in bold the patients with the most severe phenotype).

	Sex	Age (year)	Spleen enlargement	Liver enlargement	Thal-like bone modifications	Hb (g/dL)	MCV (fl)	Hb A <sub>2</sub> (%)	Hb F (%)	NRBC <sup>d</sup> (x100WBC)	α/β (ratio)	Ferritin (ng/mL)	Unconjugated Bilirubin(μmol/L)	Beta globin genotype	Number of alpha genes <sup>e</sup>
<b>Family C</b>															
II-1	M	42	+	+	+	9.0	65	4.7	19.4	-	3.1 <sup>a</sup>	750	58.1	39/A	6
II-2	F	40	-	-	-	10.5	60	4.5	6.4	-	1.9	-	24.0	39/A	5
III-1	M	25	+	+	+	9.3	58	5.7	10.5	5	3.1 <sup>a</sup>	250	66.7	39/A	6
III-2	F	18	+	-	±	8.7	59	5.9	5.3	-	3.3	135	32.4	39/A	6
<b>Family B-D</b>															
IV-2	F	55	+	+	++	7.0	58	4.2	2.4	5	4.0	120	42.7	39/A	6
IV-4	F	50	+	-	-	9.5	63	5.6	2.7	-	2.5	65	22.2	39/A	5
V-4	M	16	+	-	+	8.6	57	5.0	7.2	-	-	150	44.4	39/A	6
V-7	F	27	<b>Splenectomy</b>	+	+	7.0	61	4.4	14.8	296	2.8 <sup>a</sup>	420	39.3	39/A	6
<b>Family G</b>															
II-1	M	37	+	+	±	9.8	63	5.4	7	-	2.5	600	24.0	39/A	5
II-2	F	27	+	-	±	8.6 <sup>b</sup>	58	4.4	11	-	2.0	280	26.6	39/A	5
III-1	M	4	+	-	++	tx <sup>c</sup>	62	4.1	15.6	14	4.2 <sup>a</sup>	507	46.1	39/A	6

<sup>a</sup>α/β+γ; <sup>b</sup>Sporadic blood transfusions during pregnancy; <sup>c</sup>tx= replated red blood cell transfusion; <sup>d</sup>NRBC = nucleated red blood cells; WBC= white blood cells; <sup>e</sup>For the complete alpha globin genotype see Figure 1.

while revealed in 2 of them (IV-4 in family B-D and II-1, II-2 in family G) the presence of the -α 3.7 deletion and in a patient of family C (II-2) the presence of the α2initiation codon ATG>ACG mutation (indicated as a<sup>NcoI</sup> in Figure 1 since this mutation is recognized by the NcoI restriction enzyme). MLPA analysis of the α-globin cluster revealed in all patients with thalassemia intermedia a complete duplication of the α-globin gene cluster spanning in one family 170 Kb region from the telomere while in the other families the duplication was larger than 170kb. The duplications include the regulatory elements MCS-R1, MCS-R2(HS40), MCS-R3 and, MCS-R4. This genotype was also present in subject I-2 in G family with normal hematologic parameters (MCV 80 fL, HbA<sub>2</sub> 2.9%, Hb 15.2 g/dL and HbF 0.9%) but globin chain synthesis imbalance (α/β 1.9)

This study describes a group of patients belonging to four Sardinian families, simple heterozygotes for β thalassemia with a variable thalassemia intermedia phenotype. We have previously reported these families, postulating the presence of an unknown silent molecular defect unlinked to β cluster interacting with the β globin gene mutation.<sup>12,13</sup> Similar cases have been described by Faà *et al.*<sup>22</sup> Using the high resolution MLPA method we have been able to identify the reason for the worsening of the symptoms in the simple β thalassemia carriers of our families. In fact, beside the heterozygosity for β thalassemia, they have a duplication of the α globin locus and some of them a coexistent deletional or non-deletional α thalassemia. The duplications spanning at least 170 Kb include all the regulatory elements of the α globin gene cluster. The final result of the α globin cluster rearrangements is the presence of one or two additional α globin genes, which cause a more severe imbalance of the α/β globin chain synthesis ratio, as compared to the simple β thalassemia carrier state. The increased α/β imbalance enhances the ineffective erythropoiesis, resulting in evi-

dent clinical symptoms. It is interesting to note that the severity of the clinical phenotype seems to be partly related to the number of the α globin genes and to the type of interacting α thalassemia. In fact subjects with six α globin genes have the highest α/β ratio (3.1–4.2) and the most severe phenotype, while subjects with duplication and interacting α thalassemia, and hence with five functional α genes, have a milder phenotype and a milder α/β ratio (2.0–2.5). However, among the subjects with six α globin genes 3 have a more severe and 4 a milder clinical phenotype. The reasons of this interfamilial (family C versus family B-D and G) and intrafamilial (V-4 versus IV-2 and V-7 in family B-D) variability have not been defined. However, the individual variability could be related to the ubiquitine proteolytic pathway, the process which eliminates the excess of unbounded globin chains in the cell, previously postulated as being variable from person to person in the same family.<sup>23</sup> In this respect subject II-2 from family C, who has a non-deletional α thalassemia defect, known to be more severe than the deletional α thalassemia and therefore able to further reduce the α/β imbalance, presents the lowest α/β ratio (1.9) and the mildest phenotype, not very different from a simple β thalassemia carrier state, except for a more pronounced anemia. From these data it seems that the critical threshold of globin chain imbalance above which clinical symptoms appear is an α/β ratio of 2.0. It is interesting to note that the subject with only α globin gene duplication (I-2 in G family) shows a silent hematologic phenotype and normal HbA<sub>2</sub> but imbalanced α/β ratio, further proving that the extra α globin genes are functional.

It is well known that the presence of increased α globin chain production in heterozygotes for β-thalassemia, as a result of co-inherited triple α globin arrangement, converts the typical clinically asymptomatic β thalassemia carrier state into that of thalassemia intermedia.<sup>8-10,24</sup> Other α glo-

bin gene rearrangements associated with  $\beta$  thalassemia carrier state and resulting in thalassemia intermedia, have been reported.<sup>25</sup> However, the phenotype of the patients with these genetic combinations is variable and depends both on the severity of the  $\beta$  thalassemia allele and on the number of excess  $\alpha$  globin genes. Recently Hartevelde *et al.*<sup>16</sup> described segmental duplications of the  $\alpha$  globin gene cluster causing thalassemia intermedia in 2 subjects heterozygotes for  $\beta$  thalassemia. One patient with the typical thalassemia intermedia phenotype, had six active  $\alpha$  globin genes, as a result of duplication, the other with a transfusion-dependent phenotype had a duplication of the  $\alpha$  locus in one chromosome and the common anti- $\alpha$  3.7 triplication in trans resulting in a total of seven active  $\alpha$  genes. Globin chain synthesis analysis was not reported in this study. In our families, 3 of the subjects with six  $\alpha$  globin genes have a more severe phenotype the reasons for which have not been clarified and the presence of another not yet identified genetic determinant cannot be excluded. Since families are originating from distant areas and the exten-

sion of the duplication seem to be different, at least in one family,  $\alpha$  globin gene quadruplication is expected to be a not uncommon defect.

In conclusion, we have demonstrated that  $\alpha$  globin cluster duplications should be considered in cases of thalassemia intermedia phenotypes presenting with simple heterozygosity and in carriers of suspected silent  $\beta$  thalassemia. The reported duplication and other similar defects could possibly not be uncommon in Sardinia and elsewhere.

## Authorship and Disclosures

RG wrote the paper and takes primary responsibility for it. MCS and MEP performed the laboratory work and contributed to writing the paper. LP and DL performed the laboratory work and participated in the analysis of the data. NG recruited the patients and collected the clinical data.

The authors reported no potential conflicts of interest.

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