Asymptomatic and mild β-thalassemia in homozygotes and compound heterozygotes for the IVS2+1G>A mutation: role of the β-globin gene haplotype

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Background and Objectives. We report on two families in which the β^0-thalassemia mutation IVS2+1G→A occurs either in the homozygous or compound heterozygous condition with other β-thalassemia determinants. In the first family the proband, homozygous for the IVS2+1 determinant, is asymptomatic and was detected by chance during a screening program for β-thalassemia. In the second family, the proband is a 43-year old female with a very mild thalassemia intermedia due to compound heterozygosity for the IVS2+1G>A and IVS1+110G>A mutations. Her father was diagnosed as having a thalassemic disorder only during the family studies carried out because of the proband's condition. He is a compound heterozygote for the Sicilian type $\delta\beta^0$-thalassemia and the IVS2+1 mutation and has a normal level of hemoglobin.

Design and Methods. In both families, the heterozygous carriers of the IVS2+1G>A have unusually elevated levels of fetal hemoglobin (HbF), and the homozygotes showed 98% HbF, reflecting an increased production of well hemoglobinized F-cells not associated with a significant erythroid expansion.

Results. The high HbF levels co-segregate with the β^0-thalassemia mutation; the size and structure of both pedigrees do not allow the contribution of unlinked genes to the elevated production of HbF to be assessed.

Interpretation and Conclusions. We propose that the unusual phenotypes resulting from homozygosity and compound heterozygosity for IVS2+1 are, against the background of a polygenic quantitative control of HbF expression, principally due to elements, such as repetitive sequences or single nucleotide polymorphisms, within or closely linked to the β-gene cluster. These are potentially implicated in chromatin environment modifications, and could, therefore, be responsible for sustained HbF synthesis during development.

Key words: thalassemia intermedia, β-thalassemia mutations, fetal hemoglobin, β-globin gene cluster polymorphisms.

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The clinical presentation of β-thalassemia is highly variable, ranging from severe, transfusion-dependent anemia to milder conditions known as thalassemia intermedia; the disorder can also, in rare cases, remain asymptomatic for years.

The patient's phenotype is determined by the β-chain deficiency and α/β globin imbalance and is influenced by a variety of genetic factors linked or unlinked to the β-globin cluster. The variable severity of β-thalassemia alleles (from β^0 to β^+, β^{++} or even β^{silent}), sustained fetal hemoglobin (HbF) production and the co-inheritance of α-thalassemia are the three main factors that modify the phenotype.[1]

The only apparent explanation of the mild phenotype in some individuals who are homozygous or compound heterozygous for β^0 thalassemia is the persistent synthesis of γ-globin in adult life.

The genetic analysis of a number of thalassemic as well as non-thalassemic pedigrees has led to the discovery of loci unlinked to the β-gene cluster that have a major effect on the heterocellular expression of fetal hemoglobin in the adult.[2-7] In other normal individuals as well as in carriers of β-thalassemia or HbS, the increased γ-chain production is genetically determined and partially associated with β-haplotypes characterized by the presence of particular microsatellite sequences and/or the X*mn*I/Gγ polymorphism.[8-9]

We propose that the unusual phenotypes found in our two families are mainly due to the contribution of *cis*-acting elements to the production of HbF.

This suggestion is supported by the following observations: (i) in some populations the IVS2+1G>A mutation (and a number of other β^0-thalassemia determinants), when linked to an haplotype carrying the polymorphic X*mn*I restriction site 5' to the Gγ gene, are represented at a very significant excess in the genotypes of people with mild thalassemia intermedia; (ii) the same thalassemia mutation(s), on the background of other X*mn*I negative haplotypes, usually cause a disease with a severe clinical course.[10-11]

In both pedigrees the extended β-cluster haplotype identified in *cis* to the IVS2+1 mutation includes single nucleotide polymorphisms (SNPs) and polymorphic microsatellites which have been singly associated with elevated expression of HbF.

Design and Methods

The Thalassemia Center of Catania referred the two Sicilian families selected for this study. The probands

Table 1. Extended β-haplotypes, including RFLP and microsatellites, segregating in both pedigrees analyzed.

	A *βIVS2+1G>A*	*B* *βIVS1+110*	*C* *Sic. δβ⁰*	*D* *β^A Hapl. III*	*E* *β^A Hapl. I*	*F* *β^A Hapl. I*
HS_2 β-LCR	$(AT)_9(AT)_{10}$	$(AT)_9(AT)_{11}$	$(AT)_9(AT)_{11}$	$(AT)_9(AT)_{10}$	$(AT)_9(AT)_{11}$	N.D.
Hinc II ε	_	+	+	_	+	+
5'Gγ -158	T	C	C	T	C	C
Hind III Gγ	+	_	_	+	_	_
Gγ IVS2*	GA	GE	GG	GA	GB	GE
Hind III Ag	_	_	_	_	_	_
Aγ IVS2*	AA	AC	AM	AA	AC	AC
Hinc II ψβ	+	_	_	+	_	_
Hinc II ψβ 3'	+	_	_	+	_	_
Hinf I 5'β	_	+		_	+	+
Rsa I 5'β	+	_		+	_	+
Silencer 5'β	$(AT)_9T_5$	$(AT)_7T_7$		$(AT)_9T_5$	$(AT)_7T_7$	$(AT)_7T_7$
Ava II β	+	+		+	+	+
Rsa I 3'β	_	+		_	+	+

**Gγ and Aγ IVS2 $(TG)_n$ polymorphisms were named according to Lapoumeroulie et al. 1999.[17]*

(case I-1 and II-2 of families G.L. and S.A., respectively, Figure 1) are part of a series previously described by Ragusa *et al.*[12-13] Red cell indices, HbA_2 and HbF levels as well as determination of F-cells, β-haplotype and β-globin gene mutation analysis were performed using standard protocols.

Molecular characterization of the α-globin gene defects was carried out by DNA analysis as reported by Fichera *et al.*[14]

Segregation of multi-allelic polymorphisms $(AT)_xN_{12}(AT)_y$ of HS2 β-LCR, $(TG)_n(CG)_m$ repeat region within the IVS2 of the Gγ and Aγ genes and of the sequence $(AT)_x T_y$ upstream of the β-gene were analyzed using the procedures described by Ragusa *et al.*,[13] Perichon *et al.*[15] and Lapoumeroulie *et al.*[16] Repeat motifs of each polymorphic locus in heterozygous individuals were verified by sequencing cloned polymerase chain reaction products as described by Lapoumeroulie *et al.*[16]

Results

The first family (G.L.) was discovered during a β-thalassemia screening program involving school children. The propositus, a 14-year old boy at the time, was asymptomatic and showed normal growth. His total Hb was within normal limits (12 g/dL) but consisted of 98% HbF an 2% HbA_2. The parents had never noticed any sign suggesting anaemia. On clinical examination, however, the spleen was palpable 3 cm below the costal margin.

Analysis of the propositus' DNA revealed homozygosity for the mutant IVS2+1G>A which causes abnormal mRNA splicing and results in $β^0$ thalassemia. Both parents and the other three siblings are heterozygous for the same mutation and have a typical thalassemic blood picture with microcytosis, high HbA_2 and unusually elevated HbF levels ranging from 3.4 to 14.0% (Table 1 and Figure 1).

The extended haplotypes (including restriction fragment length polymorphisms and microsatellites) of all family members are presented in Table 1. The IVS2+1G>A mutation is in *cis* to β-globin haplotype III (here indicated as A) positive for the XmnI restriction site at position -158 upstream of the Gγ gene; the other two haplotypes (E and F) are variants of haplotype I and are in *cis* to the normal allele $β^A$. Recent hematologic evaluation of the propositus showed that the blood picture had not differed significantly from that observed at the moment of diagnosis (Hb 12.4 g/dL, Figure 1).

In the second family (S.A.), along with the IVS2+1G>A, two more thalassemic determinants, IVS1+110G>A and the Sicilian type $δβ^0$ thalassemia segregate in three generations. The former mutation results invariably in a transfusion-dependent β-thalassemia major in homozygotes, whereas the latter is a 13.377 bp deletion which includes the third exon of the δ gene and the whole β-gene and is responsible for thalassemia intermedia in homozygotes and for mild anemia and elevated HbF (4-19%) in heterozygotes.

The hematologic and molecular data of the family are presented in Table 1 and Figure 1. The propositus (II-1) was a 43-year old female, affected by a very mild form of thalassemia intermedia caused by compound heterozygosity for the IVS2+1 and IVS1+110 β-globin gene mutations.

The patient's clinical condition is so mild that the diagnosis was made only when she was already 34 years old. Since then the patient has been trans-

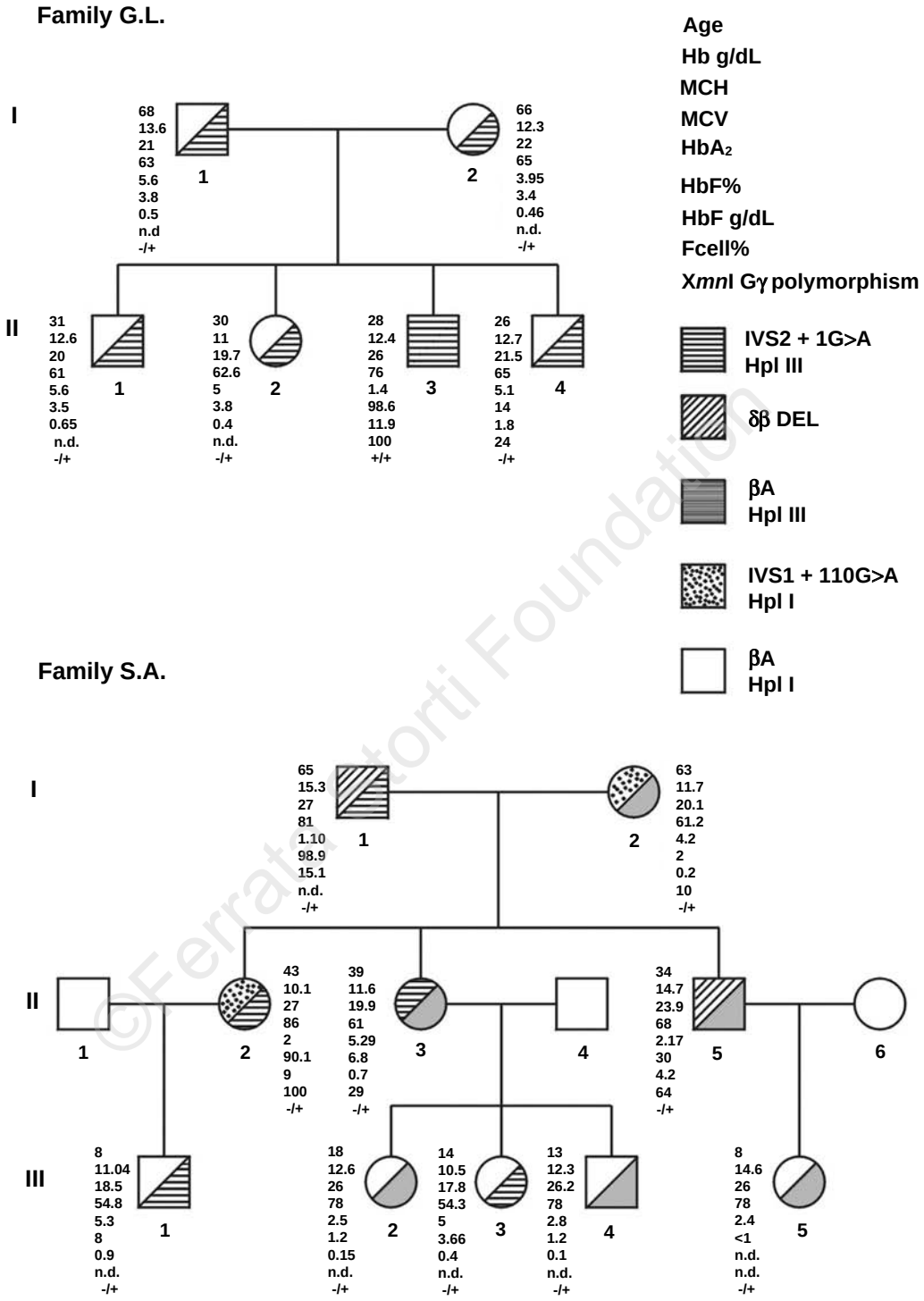

Figure 1. Pedigree of families G.L. and S.A.

fused only twice.

The father (I-1) is a compound heterozygote for Sicilian $\delta\beta^0$ thalassemia and the IVS2+1 splice mutation. His genotype was first characterized during the family study carried out because of the proband's condition. Until then he had remained asymptomatic despite a large splenomegaly. The hematologic analysis showed a Hb of 15 g/dL, consisting almost exclusively of fetal hemoglobin. The mother (I-2) is heterozygous for the IVSI+110 and shows the characteristic blood picture of thalassemic trait.

Five β-haplotypes segregate in the pedigree (Table 1). The IVS2+1 determinant (A) is associated with a haplotype III identical to that identified in family G.L., the IVS1+110 mutation (B) is located in *cis* to haplotype I, the Sicilian $\delta\beta^0$ thalassemia (C); the normal β^A allele is in *cis* either to haplotype III (D) or haplotype I (E).

HbF levels range from 2 to 8% in the heterozygous carriers of β-thalassemia and up to 30% in the individual carrier of $\delta\beta^0$ thalassemia in whom 64% of F cells were found (Figure 1). The ratio Gγ/Aγ ratio is higher than 1 (59-63% Gγ) in all members of the family.

α thalassemia, as well as deletions or single base substitutions of the β globin cluster leading to hereditary persistence of fetal hemoglobin, were excluded in both families.

Discussion

The striking aspect about the patients of these two families is the asymptomatic phenotype of homozygosity for the IVS2+1 mutation and the extremely mild thalassemia intermedia presented by the two compound heterozygotes for the same mutation and either $\delta\beta^0$ thalassemia or the severe IVS1+110 determinant.

The unusual clinical picture of the three individuals is clearly related to the high capacity of the bone marrow to produce a sufficient number of well hemoglobinized F-cells without major signs of ineffective erythropoiesis and erythroid expansion. Several structural changes of the γδβ-complex could account for the beneficial increase of fetal Hb levels observed in the thalassemic individuals of both pedigrees.[17] Besides the Sicilian $\delta\beta^0$ mutation, we did not find any other deletion which might cause hereditary persistence of fetal hemoglobin (HPFH). Mutations of γ-genes promoters were ruled out by sequencing. It is, therefore, quite likely that the elevated production of HbF in the thalassemic individuals studied is caused by the co-inheritance of β-thalassemia and a form of HPFH. This heterogeneous group of conditions is characterized by a small or moderate increase of HbF and F-cells in the normal individual, but is responsible for a marked effect following erythropoietic stress. The capacity for elevated Hb F production has been attributed to single nucleotide or microsatellite polymorphisms in *cis* to the β-gene cluster as well as to the action of genes or regulatory elements located on other chromosomes.[17] A polygenic control of fetal hemoglobin is well documented and three quantitative trait loci (QTL) have been mapped to chromosomes X, 6 and 8.[6,18-19]

In both families presented here, the high HbF levels co-segregate with the IVS2+1 mutation in *cis* to the β-haplotype III, involving the presence of the restriction site X*mn*I site (C>T at -158 5' to the Gγ gene cap site), described as being associated with increase of HbF and F-cell levels, delayed GγAγ switching and a fetal type Gγ/Aγ ratio (60-70 Gγ/40-30 Aγ) in the residual fetal Hb present in the adult.[19-23]

The same X*mn*I+ haplotype, in *cis* to the normal β^A gene, segregates in family S.A. and is found in the heterozygous condition in three normal individuals and in a carrier of $\delta\beta^0$ thalassemia. Two out of the three normal individuals show HbF percentages higher than 1%. Considering their age this finding might not be significant; the high percentage of HbF (30%) and F-cells shown by the heterozygote for $\delta\beta^0$ thalassemia is, however, remarkable.

To date, two other thalassemic families with IVS2+1 mutation have been reported. However, in the first case the mutation was located on haplotype I and the high fetal Hb production was limited to only one of the two homozygotes carrying the same mutation on an identical haplotype. In the second one, the mutation was in *cis* to different hybrid X*mn*I-haplotypes, and the pedigree included non-thalassemic individuals with higher than normal numbers of F cells.[4,25]

In the Sicilian families, polymorphic microsatellites were further identified within the haplotype carrying the IVS2+1 mutation: $(AT)_9N_{12}(AT)_{10}$ in the 3' region of the β-LCR/HS_2, a $(TG)_{13}$ sequence in the Aγ gene IVS2, in *cis* with a $(TG)_{11}(CG)_3$ sequence in the Gγ gene IVS2, and finally the $(AT)_9T_5$ motif 0.5 kb 5' to the β-gene.

All these polymorphic variants have been singly associated with an increased production of HbF but to the best of our knowledge these polymorphisms have never been found associated in a unique haplotype.

The frequency of the β^0-thalassemia allele IVS2+1G>A is rather low in Italy and Greece (about 2%), higher in the Middle East (7 to 47%), Azerbaijan (21%), and in the African population of Guadeloupe (15%).[26] The mutation can be linked to various β haplotypes (I, III, IX or *atypical*) and results in a disease of variable severity. However, very mild to moderate phenotypes are virtually only found among individuals who are homozygotes for the mutation

in cis to the X*mn*I+ haplotype III.[10-11,27] The same observation holds also for other β^0 determinants such as Cd 6 (-A), Cd 8 (-AA) and Cd 30 (G>C). These mutations, when in *cis* to X*mn*I+, often result in a mild or moderate anemia in homozygotes, whereas X*mn*I negative determinants, such as Cd 39 (C>T) or IVS1+110G>A, frequent in the Middle East, almost invariably cause severe Cooley's anemia.

In Sardinia mild thalassemia occurs in homozygotes for Cd 39 (X*mn*I-) as well as compound heterozygotes for Cd 39 and the X*mn*I+ Cd 6 (-A).[28] Family studies suggest the involvement of an unlinked HPFH determinant in many cases.[29]

In Greece a measurable (1.7-9.0%) amount of HbF is found only in heterozygote carriers of the X*mn*I+ haplotype IIIa.[30] These findings seem to restrict the propensity to produce higher levels of HbF to a specific X*mn*I + haplotype III.

Although a correlation between high HbF production and the Gγ-X*mn*I site was demonstrated in 1985, the functional significance of this has never been documented so far.[20]

Conversely, several studies in different genomic environments, propose that multiallelic microsatellites polymorphisms play a functional role in quantitative control of gene expression and thus of phenotypic variation.[31-33]

In particular for the β-gene cluster, binding of a *trans*-acting factor (BP1) to a silencer sequence 5' to the β-globin gene was shown in 1989 by Berg *et al.*[34] A variation in this $(AT)_xT_y$ polymorphism was later proven to correlate in AS individuals with decreased expression of the βS-globin gene and increased expression of the γ-globin genes.[35]

The gene coding for the BP1 protein, mapped on chromosome 17, is an isoform of DLX4, a homeobox gene involved in developmental processes.[36]

Several authors have reported a negative regulatory action of the AT rich zone of the H2S-LCR, $(AT)_xN12(AT)_y$ sequence.[37-42] The specific conformation of the H2S-LCR site observed in the carriers of the IVS2+1 mutation was shown to be associated with an increased proportion of F-cells among normal Sicilian individuals.[40]

On the other hand, a potential binding site for BP1, similar to the sequence identified upstream of the β-globin gene, has been identified in the 3' part of the $(AT)_xN_{12}(AT)_y$ core segment. It seems, therefore, of special interest that the factor binding to the H2S-LCR and the β-globin promoter region is a homeotic protein with identified repressing properties, reminiscent of another homeodomain protein with putative regulatory functions, coded by the HOXB2 gene that binds to (AT) rich regions of the LCR, the γ-globin promoter and the Aγ-globin enhancer.[43] The silencing of the β-gene could thus be balanced by an increased expression of γ-genes during erythropoietic stress. A reciprocal regulation of β and γ-globin genes has been previously documented *in vitro* and *in vivo*.[44,45]

Finally the tandem repeat $(TG)_{13}$ within the IVS2 of the Aγ-gene has been found[16] more frequently in Sicilian individuals with thalassemia intermedia than in patients with severe Cooley's anemia.

The fundamental question arising from these data is, therefore: besides the presence of the X*mn*I-Gγ site, is the association of the other polymorphisms, in particular a β-globin haplotype, relevant to the reactivation of HbF production under erythropoietic stress?

It can be hypothesized that the combination of specific recognition sites and their interaction with transcription factors could generate chromatin structures with regulatory properties (gene activation or repression).[35–37,43-46]

Our observation goes a step further, since it involves the co-existence of several microsatellite polymorphisms which have been singly proven to be the binding sites for *trans*-acting factors.

An interaction of these factors is obviously possible, and could induce chromatin modifications. Our hypothesis is that of a possible epigenetic effect due to modification of the chromatin environment. The γ genes could then entirely compensate for the β^0 thalassemic defect.

Epigenetic regulation of gene expression during development is now a well-recognized phenomenon, as is the role of DNA methylation in this feature.[47] Transcriptional de-repression has been shown to be a potential cause of genetic diseases.[48] Why not propose the same hypothesis for the expression of γ genes and the rescue of thalassemia phenotype?

The mildness of the β-thalassemic phenotype in both families presented here seems to correlate with intrinsic property(ies) of a particular haplotype. The study of the structural and functional properties of such haplotype(s) in the relevant populations will give insights into the mechanism of the benign evolution of the disease in some homozygous β^0 thalassemias.

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Contributions

AR: conception and design, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content, final approval of the version to be published; SA: conception and design of the study, analysis and interpretation of data, and drafting the article or revising it critically for important intellectual content; TL: drafting the article and revising it critically for important intellectual content; LC: conception and design, or analysis and interpretation of data; MM-R: conception and design of the study, analysis and interpretation of data; DL: drafting the article and revising it critically for important intellectual content; final approval of the version to be published; LB: drafting the article or revising it critically for important intellectual content; and final approval of the version to be published. The author thank Mr. Maurizio Sturnio from the Laboratorio di Patologia Genetica, IRCCS OASI M.SS Troina, for technical assistance during preparation of the manuscript.

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In the following paragraphs, Professor Cazzola summarizes the peer-review process and its outcomes.

What is already known on this topic

The clinical presentation of individuals who are homozygotes or double heterozygotes for β-thalassemia mutations is highly variable, ranging from severe, transfusion-dependent anemia to milder conditions known as thalassemia intermedia. The ability to produce HbF is a factor capable of modulating the clinical phenotype of thalassemic patients.

What this study adds

The unusually mild thalassemic phenotypes reported in this study appear to be mainly due to the contribution of cis-acting elements to the production of HbF.