In conclusion, in never or minimally transfused patients with thalassemia intermedia we found no evidence of cardiac iron overload, while there may be significant hepatic iron accumulation. However, since cardiac MRI has not been routinely used in TI, further studies and longer follow-up are needed to understand if and when detectable cardiac iron deposition can occur. Therefore, all patients with TI, and especially those who do receive occasional transfusions, should be evaluated regularly for cardiac and liver iron overload.

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Defective mRNA levels are responsible for a β-thalassemia phenotype associated with Hb Federico II, a novel hemoglobin variant [β-106 (G8) Leu→Val]

Hemoglobinopathies are widespread monogenic disorders that encompass complex and partially overlapping hemoglobin disorders and thalassemia syndromes. About 960 hemoglobin variants have been identified, some of which are reported to be unstable.[1] Various mechanisms for the decreased stability of a hemoglobin variant, which gives rise to differences in clinical manifestations, have been proposed. In the case of thalassemic hemoglobinopathies, structural changes are associated to quantitative defects of the corresponding globin chain, thus generating typical thalassemic phenotypes.[2]

Although it is generally agreed that clinical effects are related to an abnormal protein, it is conceivable that in some cases the globin variant may impair expression mechanisms by producing aberrant mRNA that could either be inadequately processed or be unstable possibly as a consequence of changes in the secondary structure. However, this intriguing hypothesis has yet to be demonstrated.[3]

As we previously reported,[4] during screening for couples at risk for β-thalassemia we had examined a patient presenting with hypochromic microcytic anemia and increased HbA2 level, not associated to any clinical alteration (Figure 1A). No abnormal hemoglobin fractions were observed at the cation-exchange HPLC analysis or cellulose acetate electrophoresis. Morphological analysis of red blood cells revealed anisopoikilocytosis with moderate microcytosis; erythrocytes showed increased osmotic resistance and absence of Heinz bodies. Thermal and isopropanol hemoglobin stability tests were negative although these data are not completely reliable due to the limited amount of circulating abnormal variant. Serum iron, transferrin, ferritin and bilirubin levels were normal.

Molecular analysis was performed on DNA and RNA from whole blood samples after local Ethics Committee approval and informed consent were obtained. β-globin gene sequence analysis was performed on PCR products encompassing the entire genomic sequence from 600 bp upstream from the initiation transcription site to 170 bp downstream from the termination codon, as previously reported.[5] Rearrangements in the α-globin gene cluster were excluded by Southern blotting.[6]

Sequence analysis revealed a mutation at heterozygous level in the third exon of the β-globin gene, which caused the substitution of the Leu residue with a Val residue (CTG→GTG) at codon 106, thereby producing a novel hemoglobin variant (Figure 1B). We designated this variant *Hb Federico II*.[4] No other sequence alterations were detected in the β-globin gene, which strongly suggests that this mutation is associated to a β-thalassemic trait. Hb Federico II was undetectable at cation-exchange HPLC analysis and produced a small abnormal peak (7-10% of total hemoglobin) at reverse-phase HPLC which eluted before the β-globin peak.

Because of these unusual chemical features, no functional data were available but, on the basis of computational analysis and protein modelling studies, the β 106 Leu→Val substitution is not expected to influence stability and function of the protein.

We checked for putative activation of cryptic splicing sites leading to abnormal untranslated transcripts by carrying out a computational search for splice enhancer regions (*http://rulai.cshl.edu/tools/ESE3/*) and RT-PCR analysis on full-length β-globin cDNA. In neither case were variations found with respect to normal controls (Figure 1C and D). cDNA sequencing analysis confirmed the heterozygous condition although there appeared to be a different ratio between the two mRNA species (Figure 1D, 1 and 2).

Therefore, to evaluate whether low output of this variant was correlated to reduced mRNA levels, we measured β-globin mRNA levels by real-time PCR. Total β-globin mRNA levels from the propositus's peripheral blood were comparable with those detected in carriers of frequent β-thalassemic mutations, and about three-fold lower than those of a normal subject, thus confirming a quantitative β-globin gene expression defect (Figure 2A).

Furthermore, to evaluate whether defective β-globin expression was associated to the mutant allele, allele-specific mRNA expression was determined and revealed a strong disequilibrium in the expression levels of normal versus aberrant β-globin mRNA species. In fact, levels of abnormal β-globin mRNA were about 20-fold

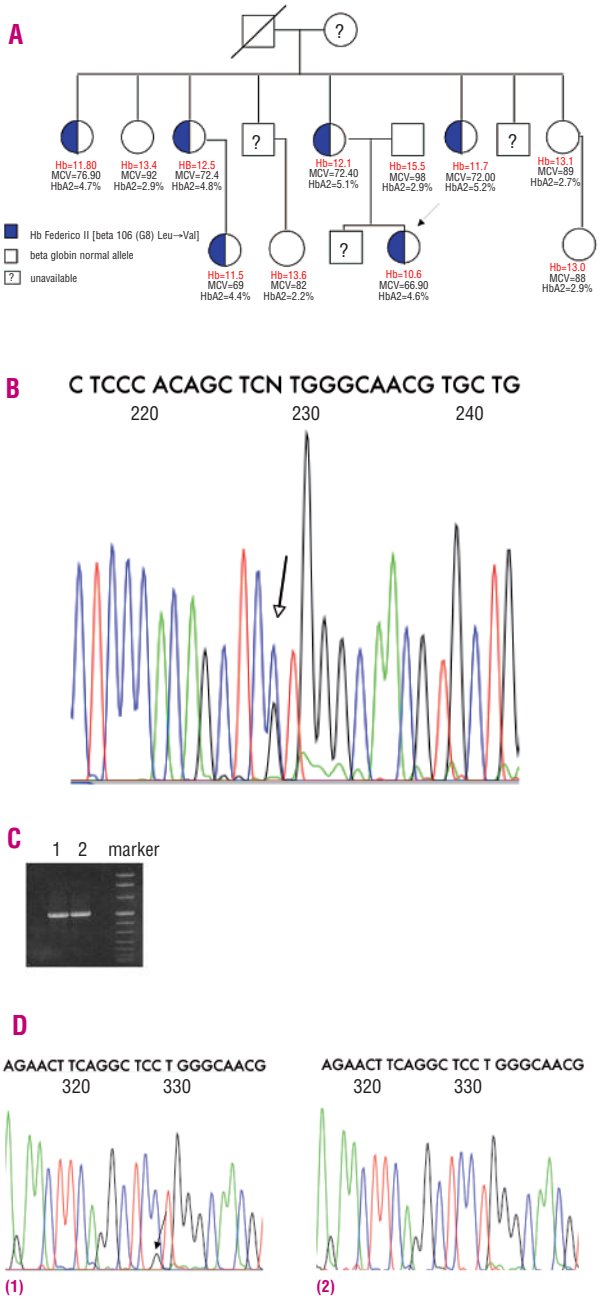

Figure 1. Detection of Hb Federico II. (A) Pedigree of the family in whom the novel variant was identified. The arrow indicates the propositus first examined. (B) β-globin genomic sequence analysis showing the heterozygous state for the mutation at codon β106 (CTG→GTG). The arrow indicates the position of mutation. (C) Gel electrophoresis of β-globin cDNA RT-PCR analysis in the propositus (1) and in a normal control (2). (D) β-globin cDNA sequence analysis showing the heterozygous state for the mutation at codon β106 (CTG→GTG) in the propositus (1) compared to a normal control (2). The arrow indicates the anomalous peak detected in the propositus.

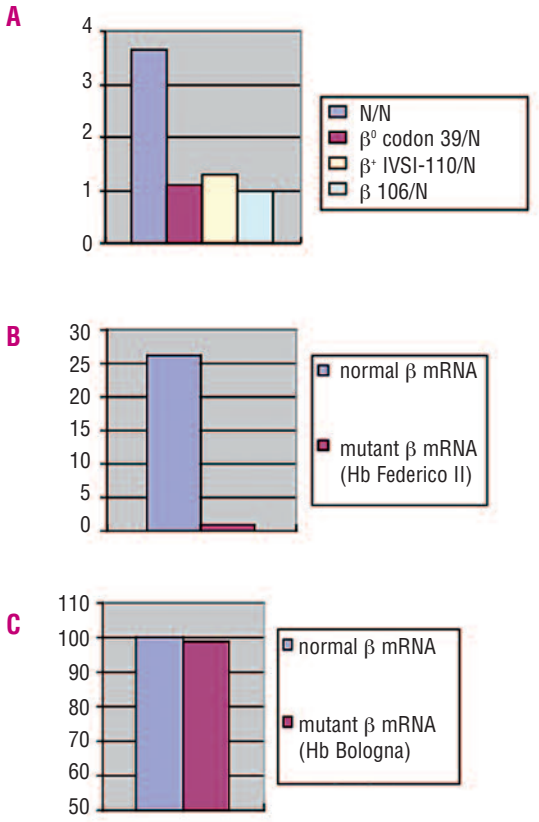

Figure 2. Evaluation of mRNA expression levels for Hb Federico II. (A) Evaluation of β-globin gene expression by quantitative real time RT-PCR analysis on total RNA isolated from peripheral blood of a normal subject, 2 carriers of typical β-thalassemia mutations, respectively β°39 and β+IVSI-110, and the propositus. (B) Relative expression of normal β-globin mRNA compared to the mutated β106 (CTG→GTG) allele evaluated by allele-specific real time PCR on total RNA isolated from peripheral blood of the propositus. (C) Relative expression of normal β-globin mRNA compared to the mutated β61 (AAG→ATG) allele evaluated by allele-specific real time PCR on total RNA isolated from peripheral blood of a subject presenting with Hb Bologna, β61 (Lys →Met) generally detected at 45-50% of total hemoglobin. In this case, as expected, the levels of expression of the abnormal mRNA were comparable to those produced by the wild-type sequence, thus confirming reliability of our analytical approach.

lower than the wild-type, which is consistent with the low circulating levels of the abnormal chain (Figure 2B). These data demonstrate that this point mutation is associated to a severe reduction of the mutant mRNA expression level, which causes a β-thalassemia phenotype by eliciting β-globin chain deficiency.

The molecular mechanisms responsible for impaired gene expression have yet to be completely clarified. However, our preliminary data on mutant and normal mRNA decay rates suggest that altered RNA instability features may be involved in this process.

Our study provides the first experimental evidence that a single nucleotide mutation within the coding region of the β-globin gene affects mRNA expression levels and causes a β-thalassemic defect. Furthermore, our data suggest that other regions besides the 3'UTR, whose role in constitutively regulation of this mechanism has been recently identified,[6,7] may contribute to the stabilization of β-globin mRNA and could, therefore, help to characterize the molecular basis of thalassemic hemoglobinopathies.

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Oligoarray comparative genomic hybridization in polycythemia vera and essential thrombocythemia

Polycythemia vera (PV) and essential thrombocythemia (ET) are myeloproliferative disorders (MPD) characterized by expansion of one or more myeloid lineages, which by definition results in erythrocytosis in PV and thrombocytosis in ET. The etiology and pathogenesis are still largely unknown.

At diagnosis, about 15% of PV and 5% of ET patients show chromosomal abnormalities.[1] The most common cytogenetic abnormalities detected by conventional cytogenetic techniques are deletions in the long arms of chromosomes 20 and 13, duplication of 1q, and trisomies of chromosomes 8 and 9.[1] Kralovics *et al.*[1] found loss of heterozygosity to be present in chromosome 9p, where *JAK2* (Janus kinase 2) resides. Its exon 14 harbors JAK2V617F mutation, recently reported to be present in more than 80% of PV and in approximately 50% of ET. In addition, JAK2 exon 12 mutation in PV2, and a thrombopoietin receptor *MPLW515L/K* mutation in ET and myelofibrosis[3] are novel mutations that have been described in *JAK2V617F* mutation negative patients.

While several novel mutations have been described in myeloproliferative disorders, some patients have normal wild type alleles, although they have a clear clinical disease and often show an abnormal growth pattern in *in vitro* colony forming assays.[4] In ET in particular, approximately 50% of the patients are *JAK2* mutation negative and only a small fraction (less than 5%) has *MPL* mutation. Of particular interest are the patients who show only spontaneous megakaryocytic growth in colony forming assays, since the mechanism and possible cytogenetic abnormalities underlying this are largely unknown. Therefore, we aimed to screen the entire genome using oligonucleotide array comparative genomic hybridization (aCGH) to detect small chromosomal alterations that cannot be revealed by conventional cytogenetic techniques.

The study comprised 36 frozen specimens from 21 ET and 14 PV patients; from one patient we had both bone marrow (BM) mononuclear cells and colonies of hematopoietic progenitors. Altogether 23 BM aspirates (11 PV and 12 ET), 8 Ficoll separated BM mononuclear cells (6 PV, 2 ET), 3 whole blood cells (1 PV, 2 ET) and 2 samples from colonies of hematopoietic progenitors (both ET) were studied. Fifteen specimens were JAK2V617F mutation negative (4 PV, 11 ET) and 20 were mutation positive (10 PV, 10 ET). PV was diagnosed according to the WHO criteria and ET as described by Jantunen *et al.*[5] All but one patient (dup1q) had a normal karyotype; for one patient the karyotype was not available. The study was approved by the local ethical review board and all participants gave their written consent.

Genomic DNA was extracted using standard non-enzymatic methods for frozen (stored bone marrow and whole blood) samples and the Puregene DNA purification kit (Gendra, Minneapolis, MN, USA) for mononuclear cells and hematopoietic progenitor colony cells. Reference DNA was extracted from pooled peripheral blood of healthy individuals. The hematopoietic progen-