Analysis of δ-globin gene alleles in the Sicilian population: identification of five new mutations

Although δ-globin gene (HBD MIM#142000) mutations have no clinical implications, co-inheritance of β- and δ-thalassemia may lead to misdiagnosis. Among 7,153 samples studied for β-thalassemia, 205 samples with lower than expected HbA levels were selected for our analysis and 183 samples (2.5%) were positive for δ-globin gene mutations. Twelve different mutations were detected, and among these five have not been previously described (HbA-Catania HBD c.8A→T, HbA-Corleone HBD c.41C→A, HbA-Ventimiglia HBD c.212C→G, HbA-Montechiaro HBD c.260C→A, and HbA-Bagheria HBD c.422C→T). This study suggests that δ-globin gene defects are very common in Sicily. Thus, these mutations need to be considered during β-thalassemia screening to avoid false negative results in the detection of at-risk couples.

Key words: δ-thalassemia, δ-globin gene variant, mutation detection, HBD gene GAP-PCR.

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The adult human hemoglobins (Hb) include HbA (α2β2) and HbA1; a tetramer of α and δ globin chains. Under normal conditions, HbA1 accounts for less than 3.3% of total hemoglobin, while in β-thalassemia carriers the percentage of HbA1 is approximately twice that in normal adults. Although δ-globin gene (HBD MIM#142000) mutations have no clinical implications, the co-inheritance of β- and δ-thalassemia may lead to misdiagnosis because HbA1 levels remain normal or low due to decreased δ-chain production. For this reason, the detection of δ-globin alleles is important in countries that have implemented a thalassemia prevention program because of a high incidence of β-thalassemia carriers.

Design and Methods

The samples were randomly collected from subjects coming to our Thalassemia Center in Palermo for a β-thalassemia trait test and from a school screening program. Among 7,153 samples studied for β-thalassemia, 205 samples were selected. The selection criteria included: (i) HbA1 levels ranging from 0.5% to 2.2% and normal hematologic parameters, and (ii) low levels of HbA1 associated with an HbA1 fraction variant or HbA1 value lower than expected according to the particular β- or α-globin gene mutation. Iron deficiency was excluded in all cases by measurement of ferritin levels. Hematologic data were obtained with an automated cell counter (Beckman ACT-diff; Coulter Corporation, Miami, Florida, USA). HbA, HbA1, HbF, and Hb variants were identified and measured by cation exchange high performance liquid chromatography (HPLC) on a Variant I system (Bio-Rad Laboratories, Richmond, CA, USA) using the β-Thalassemia Short Program provided by the manufacturer.

Genomic DNA was isolated from white blood cells by salting out extraction using standard protocols.1 Point mutations in α- and β-globin genes and in γ-globin gene promoters were analyzed by polymerase chain reaction (PCR) and sequenced by an ABI PRISM 3100 DNA Analyzer (PE BioSystems, Foster City, CA, USA) using the ABI PRISM Big Dye Terminator v 3.1 Cycle Sequencing Kit (PE BioSystems).

The most frequent Mediterranean α-globin gene deletions (α-Δ17, α-Δ2, --Medit, --20.5) were detected by the GAP-PCR method using primers 5′ and 3′ of the breakpoints.2 The δ-globin gene was analyzed directly by sequencing two amplified segments: the first fragment (641 bp) from position -124 of the CAP site to position 547 of IVS II (primers 5′ggaatgaagctattacccttgag, 3′ggtctctccacatgggtat) were used and the second fragment (762 bp) from IVS II nt 762 to nt 288 AT 3′ δ-globin gene (primers 5′gcgagggactactggcatcctgtctcctggtc, and 3′atcgtgtctttacattcctgtctcctggtc). The subjects for whom no point mutation was detected in δ-globin gene sequencing analysis were analyzed by GAP-PCR for the presence of the δ-Corfu deletion (U01317.1.g.48843_56050del7208).3 Three different primers (5′cacacatgttgctctatca/5′ggagt gaatccttt gcac/5′gtctctcaccatgggtat) were used to obtain an abnorme fragment of 800 bp
from the deleted chromosome and a control fragment of 644 bp from the normal allele. The γ globin genotype was studied only in samples with HbF values > 2%.

**Results and Discussion**

One hundred and eighty-three (2.5%) out of the 7,153 subjects studied were positive for a δ-globin gene defect. In 19/183 cases (Table 1) a HbA2 variant was shown on HPLC. We detected seven previously known mutations (HbA2-Mitsero, HBD c.14C→T, HbA2-NYU, HBD c.39T→A, HbA2-Yialousa, HBD c.428C→T, and -globin genotypes (Table 1) and one had an associated high HbF level (type D) (Table 1). Thirty-three also had α- and/or β-globin gene defects (Table 1). In 15 subjects, α-thalassemia was co-inherited: five with trait -α3.7 (HbA2 1.8%±0.2), two with -α2.4 (HbA2 1.6% and 1.7%), two with -α2.3 (HbA2 1.4% and 1.5%), two with -α2.1 (HbA2 1.7% and 1.9%), and two with α2.1 (HbA2 1.5% and 1.9%) (Table 1). In 15 subjects, HbA2-Yialousa was associated with β-globin gene defects: five cases with sickle cell trait (HbC 40%±0.2; HbA2 2.7%±0.1), four with IVS1 nt 6 (c.92+6T→C) (HbA2 3.0%±0.2), two with IVS1 nt 110 (c.123G→A) (HbA2 3.4% and 3.3%), one with codon 59 mutation (c.118C→T) (HbA2 3.5%), one with IVS1 nt 1 (c.128+1G→T) (HbA2 3.5%), one with a promoter mutation at nucleotide -87 (c.137C→G) (HbA2 4.6%) and one with a promoter mutation at nucleotide -101 (c.151C→G) (HbA2 2.8%) (Table 1). In four cases, HbA2-Yialousa was found in association with
-100 CAP site ambiguity T→C (HBB c.-150T→C) in cis to the δ-globin gene mutant. In one case, HbA2-Yialousa was associated with the -158 γγ (HBG2 c.-211 C→T) mutation and showed normal red cell parameters, increased HbF, and a very low level of HbA2 (Table 1).

Ten subjects (5.5%) were heterozygous for HbA2-NYU, a hemoglobin variant (Table 1). In these subjects the mean values of normal and variant HbA2 were 1.5%±0.2 and 1.6%±0.2, respectively (Table 1). Three of these cases were associated with the -100 CAP (T→C) polymorphism. In one case, HbA2-NYU was linked with the -100 CAP polymorphism, -αδ, and Hb Valletta (HBB c.262 A→C, β cd 87 ACA→CCA) (Table 1). In this case, the levels of HbA2 and HbA2-NYU were similar to those detected in the simple heterozygous state for HbA2-NYU (HbA2: 1.6%; HbA2-NYU: 1.8%) (Table 1).

An αG→αG change at the consensus 3′-acceptor site of IVS-II (δ-thalassemia) was identified in eight (4.3%) unrelated subjects (Table 1). Four were heterozygous for this mutation (HbA2: 1.5%±0.2) (Table 1). In three subjects, heterozygosity for IVS1 nt 110 (c.93 21G→A) (HbA2: 3.3%±0.2) was present (Table 1), while in one subject αωααωααωω was also detected (Table 1). HbA2-Mitsero, a δ-thalassemia genotype, was found in four cases (Table 1). In two cases this globin gene defect was linked with the neutral δ cd 97 CAC→CAT mutation, as previously described in Greek-Cypriots (Table 1). Moreover, we found a case in which HbA2-Mitsero, δ cd 97 CAC→CAT, and -αδ were inherited (HbA2-Mitsero: 1.6%) (Table 1).

One patient with an HbA2 percentage of 1% had the HbA2-Coburg mutation (Table 1). We were not able to find any HbA2-variant peak because of the co-migration of HbA2-Coburg with HbA2. Finally, three cases of HbA2-Fitzroy (HbA2: 1.4%±0.2; HbA2-variant: 1.0%±0.1) and three cases of the 7.2-Kb δ-Corfu deletion (HbA2: 1.4%±0.1) were detected (Table 1).

**Unknown mutations**

Five new mutations were discovered in unrelated families. These subjects showed normal hematologic parameters except for the reduction in HbA2 level and/or the presence of a variant peak (Figure 1). These new mutations were named according to the town origin of the carriers.

**HbA2-Catania.** A δ-globin variant of Cd 2 (HBB c.3A→T), this mutation results in the substitution of the amino acid His with Leu. The sample with HbA2-Catania had a reduced level of HbA2 (1.2%) with a Hb-variant value of 0.7% (Table 1). This variant elutes after HbA2 in the S-window zone (Figure 1).

**HbA2-Corleone.** This mutation (HBB c.41C→A) showed a low peak moving slower than HbA2 in the C-window and a HbA2 value as about 1.5-1.6% (Table 1; Figure 1). HbA2-Corleone results in the substitution of the amino acid Ala with Asp at an external position in the helix. This mutation was found in two unrelated subjects from different villages. The name of the variant was assigned according to the town of the first characterized carrier. The 5′sub-haplotype V+(+ - - - -) (RFLP HindIII/epson, Hind III/αγ, HindIII/γγ, HindII/ββ, HindII/βγ, HindII/5′β), as suggested by studies of the relatives, was associated with HbA2-Corleone in both cases.

**HbA2-Ventimiglia.** This mutation (HBB c.212C→G) resulted in the substitution of Ala with Gly at helical position E14, which is in contact with heme. HPLC analysis showed two slow fractions: the normal HbA2 with a value of 2%, and the minor slower peak variant HbA2-Ventimiglia with a value of 0.6% (20% of the total HbA2). The HbA2-variant peak eluted after normal HbA2 (Figure 1).

**HbA2-Montecliano.** This δ-globin gene defect (HBB c.260C→A) is due to substitution of Gln with Lys at helical position F3, an external position. The value of HbA2 was 1.3%, and the HbA2-variant, eluted after HbA2 in the S-window zone, was present at a similar percentage (1.5%) (Table 1).

**HbA2-Bagheria.** This variant (HBB c.422C→T) resulted in the substitution of the amino acid Ala with Val. The HbA2 fractions estimated by HPLC (Figure 1) showed 1.7% normal HbA2 and 0.5% HbA2-Bagheria.

It is evident from these findings that the most common δ-globin gene defect in Sicily is HbA2-Yialousa (81%), as was observed in other Mediterranean countries, particularly Sardinia and Greece. Moreover, a few delta-molecular defects (HbA2-Yialousa, HbA2-NYU, and IVS II, 3′) account for more than 90% (167/182) of the overall δ gene defects, as we previously described for β-thalassemia. The levels of HbA2 according to the δ-genotype are shown in Table 1. HbA2 values ranged from 1.3% to 2.0% whether δ, δ, or a variant not interfering with the normal peak of HbA2 was detected. The interaction between δ- and β-thalassemia was present in many cases with normal values of HbA2 (Table 1). The suspicion of an interaction between δ- and β-thalassemia may arise when microcythemia with borderline levels of HbA2 is detected without any findings of iron deficiency. It is useful to remember that the reduction in HbA2 levels with microcythemia may result in an α-thalassemia or iron deficiency-like phenotype, leading to an incorrect diagnosis. The mean value of HbA2-Yialousa was 1.7%±0.2. The present study suggests borderline levels of HbA2 in the presence of severe β- or δβ-thalassemia defects and normal levels of HbA2 with mild β-thalassemia defects such as IVS1 nt 6 (Table 1). It is worth noting that only in the case of β mutation -87 (HBB c.-137) with HbA2-Yialousa had an increased HbA2 value (Table 1). Two particular cases resulting in normal phenotypes are the co-inheritance of Hb A2-Yialousa with the β silent mutation -101 C→T (HBB c.151) and the change at the consensus 3′-acceptor site of IVS-II δ with αωααωααωω (Table 1). The interaction of δ-globin gene defects with α-thalassemia leads to a mild reduction in HbA2 levels (ranging from 1.6 to 2.0%) in carriers with HbA2-Yialousa and –αδ (Table 1) and to a major reduction in carriers with α deletions or point mutations in the α-globin.
gene (Table 1). The association of HbA-Yialousa with the -156 γ was characterized by a very low level of HbA (Table 1), probably due to the increased affinity of γ globins to it in comparison to the δ-variant chains, as was suggested by Bunn and Forget. In the Figure 2 we present a possible flowchart for detecting these δ-globin gene defects in our population, considering the presence of one predominant mutation (HbA-Yialousa) and the heterogeneity of the other molecular lesions. Although our data do not reflect the real epidemiology of δ molecular defects in Sicily due to the selection criteria used for detection, they do suggest that δ-globin gene defects are very common on our island, raising the possibility of misdiagnosing the diagnosis of β-thalassemia carriers. In cases with HbA-variants, it is important to consider the HPLC chromatogram carefully to avoid an incorrect diagnosis concerning β-thalassemia. Moreover, this study indicates that the same great molecular heterogeneity shown in the βδ and αδ globin genes in Sicily is also present in the δ-globin gene alleles, suggesting that a study for δ-globin gene defects should be considered as a step in the flowchart for detection of at-risk couples in our region. Moreover, the possible influences of these globin gene defects in other populations could be better explored.

Figure 1. High performance liquid chromatographic Hbs and electrophrogram data of the new mutations. A-B-D-E, HPLC data; a-b-c-d-e, corresponding electropherograms. A-a. HbA Catania δ cd 2 CAT→CTT; B-b. HbA Corleone δ cd 13 GCC→GAC; C-c. HbA Ventimiglia δ cd 70 GCC→GGC; D-d. HbA Montechiaro δ cd 87 CAG→AAG; E-e. HbA Bagheria δ cd 140 GCC→GTC.

Figure 2. Flowchart for the detection of the most common δ globin gene defects

AG is in charge of the molecular laboratory, planned, developed and supervised the experimental work and wrote the manuscript. CP, a molecular biologist, performed most experiments, designed the tables and figures and co-wrote the manuscript. GR, PT, MA, in charge of this study. They collected samples and contributed to the analysis. DR, hematologist, was consultant in this study; AM, head of U.O Hematology II and of the laboratory, coordinated the study and supervised the writing.

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