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

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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 $HbA_2$ 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 not previously described ($HbA_2$-Catania HBD c.8A→T, $HbA_2$-Corleone HBD c.41C→A, $HbA_2$-Ventimiglia HBD c.212C→G, $HbA_2$-Montechiaro HBD c.260C→A, and $HbA_2$-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 ($\alpha_2\beta_2$) and $HbA_2$, a tetramer of α and δ globin chains. Under normal conditions, $HbA_2$ accounts for less than 3.3% of total hemoglobin, while in β-thalassemia carriers the percentage of $HbA_2$ 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 $HbA_2$ 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) $HbA_2$ levels ranging from 0.5% to 2.2% and normal hematologic parameters, and (ii) low levels of $HbA_2$ associated with an $HbA_2$ fraction variant or $HbA_2$ 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, $HbA_2$, 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 ($-\alpha^{3.7}$, $-\alpha^{4.2}$, $--^{Med}$, $--^{20.5}$) 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 5' of the CAP site to position 547 of IVS II (primers 5'ggaatgaaggttcatttttcatt/5'aagtgaagcatctcctggac), and the second fragment (762 bp) from IVS II nt 762 to nt 288 AT 3' δ-globin gene (primers 5'ctgatgggaataacctgggat/5'atcgtagctattacccttgag). 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-Corfù deletion (U01317.1:g.48843_56050del7208).[3] Three different primers (5'cacacatgtgtgcattcata/5'ggggtgaattccttgccaa/5'gtctctccacatgggtat) were used to obtain an abnormal fragment of 800 bp

**Table 1.** Levels of $HbA_2$ in δ-thalassemia, in δ-globin gene variants, and in cases of interaction between δ mutations and α- or β-globin gene mutations. All of the δ alleles are in the heterozygote state except for three homozygous (*) cases. See the text for the explanation of types A, B, C and D of $HbA_2$-Yialousa.

| *Genotype δ* | *Genotype β* | *Genotype α* | *Genotype γ* | *$HbA_2$ %* | *$HbA_2$ var.%* | *Hb F %* | *No. of carriers* |
|---|---|---|---|---|---|---|---|
| | $\beta^A/\beta^A$ Type A | αα/αα | | 1.7±0.2 | | | 113 |
| | $\beta^A/\beta^A$ Type B | αα/αα | | 2.1±0.1 | | | 4 |
| | $\beta^A/\beta^A$ Type C | αα/αα | | 0.7±0.1 | | | 3* |
| | $\beta^A/\beta^A$ Type C | αα/αα | $^G\gamma$-158 /$^G\gamma$ | 1,1 | | 6.2 | 1 |
| | $\beta^A/\beta^{IVS\ 1\ nt110}$ | αα/αα | | 3.3-3.4 | | | 2 |
| | $\beta^A/\beta^{Cod\ 39}$ | αα/αα | | 3.5 | | | 1 |
| Hb $A_2$-Yialousa | $\beta^A/\beta^{IVS\ 1nt1}$ | αα/αα | | 3.5 | | | 1 |
| | $\beta^A/\beta^{87G}$ | αα/αα | | 4.6 | | 1.9 | 1 |
| | $\beta^A/\beta^{-101}$ | αα/αα | | 2.8 | | | 1 |
| | $\beta^A/\beta^{IVS\ 1\ nt6}$ | αα/αα | | 3,0±0.2 | | | 4 |
| | $\beta^A/\beta^S$ | αα/αα | | 2.7±0.1 | | | 5 |
| | $\beta^A/\beta^A$ | $\alpha^{-3,7}$/αα | | 1.8±0.2 | | | 5 |
| | $\beta^A/\beta^A\infty$ | $\alpha^{-20,5}$/αα | | 1.4-1.5 | | | 2 |
| | $\beta^A/\beta^A\infty$ | $\alpha^{MED}$/αα | | 1.6-1.7 | | | 2 |
| | $\beta^A/\beta^A\infty$ | $\alpha^{NcoI}\alpha$ /αα | | 1.5-1.9 | | | 2 |
| | $\beta^A/\beta^A\infty$ | $\alpha^{HphI}$ α/αα | | 1.5-1.9 | | | 2 |
| Hb $A_2$-NYU | $\beta^A/\beta^A\infty$ | αα/αα | | 1.5±0.2 | 1.6±0.2 | | 9 |
| | $\beta^A/\beta^{Valletta}$ | $\alpha^{-3,7}$/αα | | 1.6 | 1.8 | | 1 |
| Hb $A_2$-Mitsero | $\beta^A/\beta^A\infty$ | αα/αα | | 1.9±0.2 | | | 3 |
| | $\beta^A/\beta^A\infty$ | $\alpha^{-3,7}$/αα | | 1.6 | | | 1 |
| Hb $A_2$-Coburg | $\beta^A/\beta^A\infty$ | αα/αα | | 1 | | | 1 |
| Hb $A_2$-Fitzroy | $\beta^A/\beta^A\infty$ | αα/αα | | 1.4±0,2 | 1.0±0.1 | | 3 |
| Hb $A_2$-Catania | $\beta^A/\beta^A\infty$ | αα/αα | | 1,2 | 0.7 | | 1 |
| Hb $A_2$-Corleone | $\beta^A/\beta^A\infty$ | αα/αα | | 1.6-1.5 | 0.5-0.6 | | 2 |
| Hb $A_2$-Ventimiglia | $\beta^A/\beta^A$ | αα/αα | | 2 | 0.6 | | 1 |
| Hb $A_2$-Montechiaro | $\beta^A/\beta^A\infty$ | αα/αα | | 1.3 | 1.5 | | 1 |
| Hb $A_2$-Bagheria | $\beta^A/\beta^A\infty$ | αα/αα | | 1.7 | 0.5 | | 1 |
| 7.2 Kb deletion | $\beta^A/\beta^A\infty$ | αα/αα | | 1.4±0.1 | | | 3 |
| | $\beta^A/\beta^A\infty$ | αα/αα | | 1.5±0.2 | | | 4 |
| IVS II nt 897 A→G | $\beta^A/\beta^{IVS\ 1\ nt110}$ | αα/αα | | 3.3±0.2 | | | 3 |
| | $\beta^A/\beta^A\infty\infty$ | αα/αα | | 2.5 | | | 1 |

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 $HbA_2$ variant was shown on HPLC. We detected seven previously known mutations ($HbA_2$-Mitsero[4] HBD c.14C→T, $HbA_2$-NYU[5] HBD c.39T→A, $HbA_2$-Yialousa[6-8] HBD c.82G→T, IVS II nt 8974A→G HBD c.316-2 A→G, $HbA_2$-Coburg[9] HBD c.350G→A, $HbA_2$-Fitzroy[10] HBD c.428C→A, and δ-Corfù[3] U01317.1:g.48843_56050del7208) and five new δ-globin gene defects.

### *Previously known mutations*

$HbA_2$-Yialousa was found in 149 unrelated subjects: four types, A, B, C and D with normal $\beta^A/\beta^A$ genotype were found (Table 1). Among these, 113 were heterozygotes with $HbA_2$ 1.7%±0.2 (type A) (Table 1), and only four had $HbA_2$ >2.0%, (type B) (Table 1). Three were homozygotes for the mutation (type C) (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 13 subjects, α-thalassemia was co-inherited: five with trait -$\alpha^{3.7}$ ($HbA_2$ 1.8%±0.2), two with --$^{Med}$ ($HbA_2$ 1.6% and 1.7%), two with --$^{20.5}$ ($HbA_2$ 1.4% and 1.5%), two with $\alpha^{NcoI}$ ($HbA_2$ 1.7% and 1.9%), and two with $\alpha^{Hph\ 1}$ ($HbA_2$ 1.5% and 1.9%) (Table 1). In 15 subjects, $HbA_2$-Yialousa was associated with β-globin gene defects: five cases with sickle cell trait (HBB c.20A→T) (HbS 40%±0.2; $HbA_2$ 2.7%±0.1), four with IVS1 nt 6 (c.92+6T→C) ($HbA_2$ 3.0%±0.2), two with IVS1. nt 110 (c.93 21G→A) ($HbA_2$ 3.4% and 3.3%), one with codon 39 mutation (c.118C→T) ($HbA_2$ 3.5%), one with IVS1 nt 1 (c.-92+1G→T) ($HbA_2$ 3.5%), one with a promoter mutation at nucleotide –87 (c.-137C→G) ($HbA_2$ 4.6%) and one with a promoter mutation at nucleotide -101 (c.-151C→T) ($HbA_2$ 2.8%) (Table 1). In four cases, $HbA_2$-Yialousa was found in association with

–100 CAP site ambiguity T→C (HBD c.-150T→C) in *cis* to the δ-globin gene mutant. In one case, $HbA_2$-Yialousa was associated with the -158 $^{C}\gamma$ (HBG2 c.-211 C→T) mutation and showed normal red cell parameters, increased HbF, and a very low level of $HbA_2$ (Table 1).

Ten subjects (5.5%) were heterozygous for $HbA_2$-NYU, a hemoglobin variant (Table 1). In these subjects the mean values of normal and variant $HbA_2$ 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, $HbA_2$-NYU was linked with the -100 CAP polymorphism, $-\alpha^{3.7}$, and Hb Valletta (HBB c. 262 A→C, β cd 87 ACA→CCA) (Table 1). In this case, the levels of $HbA_2$ and $HbA_2$-NYU were similar to those detected in the simple heterozygous state for $HbA_2$-NYU ($HbA_2$ 1.6%; $HbA_2$-NYU 1.8%) (Table 1).

An AG→GG change at the consensus 3'-acceptor site of IVS-II ($\delta^0$-thalassemia) was identified in eight (4.3%) unrelated subjects (Table 1). Four were heterozygous for this mutation ($HbA_2$ 1.5%±0.2) (Table 1). In three subjects, heterozygosity for IVS1 nt 110 (c.93_21G→A) ($HbA_2$ 3.3%±0.2) was present (Table 1), while in one subject $\alpha\alpha\alpha^{anti3.7}$ was also detected (Table 1). $HbA_2$ Mitsero, a $\delta^+$-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).[4] Moreover, we found a case in which $HbA_2$ Mitsero, δ cd 97 CAC→CAT, and $-\alpha^{3.7}$ were inherited ($HbA_2$-Mitsero 1.6%) (Table 1).

One patient with an $HbA_2$ percentage of 1% had the HbA2-Coburg mutation (Table 1). We were not able to find any $HbA_2$ variant peak because of the co-migration of $HbA_2$-Coburg with HbA. Finally, three cases of $HbA_2$-Fitzroy (HbA2 1.4% ± 0.2; $HbA_2$-variant 1.0%±0.1) and three cases of the 7.2-Kb δ-Corfù deletion ($HbA_2$ 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 $HbA_2$ level and/or the presence of a variant peak (Figure 1). These new mutations were named according to the town origin of the carriers.

*$HbA_2$-Catania*. A δ-globin variant of Cd 2 (HBD c.8A→T), this mutation results in the substitution of the amino acid His with Leu. The sample with $HbA_2$-Catania had a reduced level of $HbA_2$ (1.2%) with a Hb-variant value of 0.7% (Table 1). This variant elutes after $HbA_2$ in the S-window zone (Figure 1).

*$HbA_2$-Corleone*. This mutation (HBD c.41C→A) showed a low peak moving slower than $HbA_2$ in the C-window and a $HbA_2$ value as about 1.5-1.6% (Table 1; Figure 1). Hb $A_2$-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 origin of the first characterized carrier. The 5'sub-haplotype $V_b$(+ - - - - -) (RFLP HincII/epson, Hind III/$^{G}\gamma$, HindIII/$^{A}\gamma$, HincII/phi-β, HincII/3'phi-β, Hinfπ/5'β), as suggested by studies of the relatives, was associated with $HbA_2$-Corleone in both cases.

*$HbA_2$-Ventimiglia*. This mutation (HBD 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 $HbA_2$ with a value of 2%, and the minor slower peak variant $HbA_2$-Ventimiglia with a value of 0.6% (20% of the total $HbA_2$). The $HbA_2$ variant peak eluted after normal $HbA_2$ (Figure 1).

*$HbA_2$-Montechiaro*. This δ-globin gene defect (HBD c.260C→A) is due to substitution of Gln with Lys at helical position F3, an external position. The value of $HbA_2$ was 1.3%, and the $HbA_2$-variant, eluted after $HbA_2$ in the S-window zone, was present at a similar percentage (1.5%) (Table 1).

*$HbA_2$-Bagheria*. This variant (HBD c.422C→T) resulted in the substitution of the amino acid Ala with Val. The $HbA_2$ fractions estimated by HPLC (Figure 1) showed 1.7% normal $HbA_2$ and 0.5% $HbA_2$-Bagheria.

It is evident from these findings that the most common δ-globin gene defect in Sicily is $HbA_2$-Yialousa (81%), as was observed in other Mediterranean countries, particularly Sardinia and Greece.[4,6,11] Moreover, a few delta-molecular defects ($HbA_2$ Yialousa, $HbA_2$ –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.[12] The levels of $HbA_2$ according to the δ-genotype are shown in Table 1. $HbA_2$ values ranged from 1.3% to 2.0% whether $\delta^0$, $\delta^+$, or a variant not interfering with the normal peak of $HbA_2$ was detected. The interaction between δ- and β-thalassemia was present in many cases with normal values of $HbA_2$ (Table 1). The suspicion of an interaction between δ- and β-thalassemia may arise when microcythemia with borderline levels of $HbA_2$ is detected without any findings of iron deficiency. It is useful to remember that the reduction in $HbA_2$ levels with microcythemia may result in an α-thalassemia or iron deficiency-like phenotype, leading to an incorrect diagnosis. The mean value of $HbA_2$-Yialousa was 1.7%±0.2. The present study suggests borderline levels of $HbA_2$ in the presence of severe $\beta^+$- or $\beta^0$-thalassemia defects and normal levels of $HbA_2$ with mild $\beta^+$-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 $HbA_2$-Yialousa had an increased $HbA_2$ value (Table 1). Two particular cases resulting in normal phenotypes are the co-inheritance of Hb $A_2$-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 $\alpha\alpha\alpha^{anti\ 3.7}$ (Table 1). The interaction of δ-globin gene defects with α-thalassemia leads to a mild reduction in $HbA_2$ levels (ranging from 1.6 to 2.0%) in carriers with $HbA_2$ Yialousa and $-\alpha^{3.7}$ (Table 1) and to a major reduction in carriers with $\alpha^0$ deletions or point mutations in the $\alpha_2$-globin

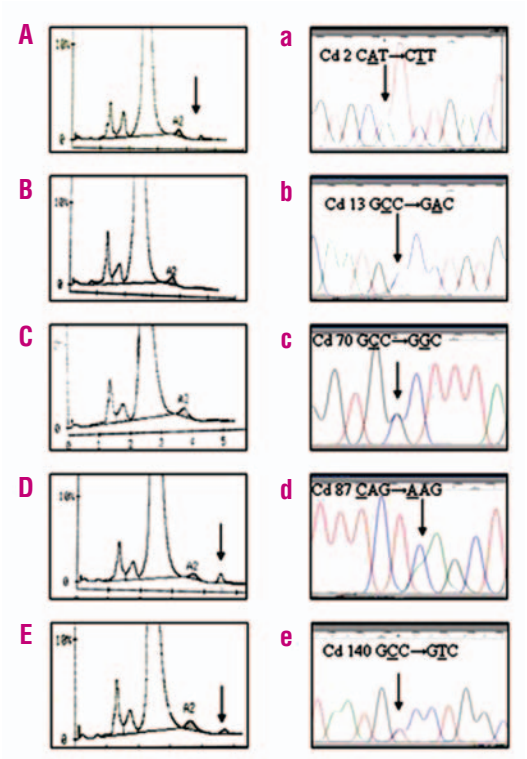


**Figure 1.** High performance liquid chromatographic Hbs and electropherogram data of the new mutations. A-B-C-D-E, HPLC data; a-b-c-d-e, corresponding electropherograms. A-a. $HbA_2$ Catania δ cd 2 CAT→CTT; B-b. $HbA_2$ Corleone δ cd 13 GCC→GAC; C-c. $HbA_2$ Ventimiglia δcd 70 GCC→GGC; D-d. $HbA_2$ Montechiaro δcd 87 CAG→AAG; E-e. $HbA_2$ Bagheria δ cd 140 GCC→GTC.

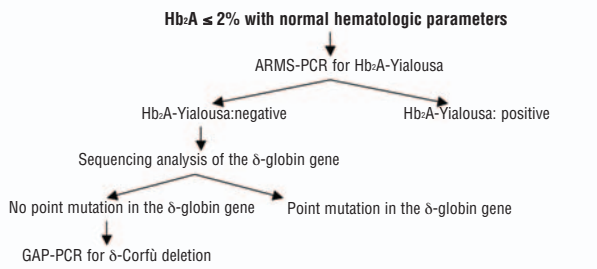


**Figure 2.** Flow chart for the detection of the most common δ globin gene defects

gene (Table 1). The association of $HbA_2$–Yialousa with the -158 $^{C}\gamma$ was characterized by a very low level of $HbA_2$ (Table 1), probably due to the increased affinity of γ globins to α in comparison to the δ-variant chains, as was suggested by Bunn and Forget.[12]

In the Figure 2 we present a possible flow chart for detecting these δ-globin gene defects in our population, considering the presence of one predominant mutation ($HbA_2$-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 misleading the diagnosis of β-thalassemia carriers. In cases with $HbA_2$ 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 β-[13] and α-[14] 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 flow chart 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.

*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 figure and co-wrote the manuscript. GR, PT, MA, was 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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