Identification and molecular characterization of a novel 163 kb deletion: The Italian ($\epsilon\gamma\delta\beta$)$^0$-thalassemia

Giovanna Cardiero¹, Romeo Prezioso¹, Sabrina Dembech², Francesca Del Vecchio Blanco³, Clelia Scarano² and Giuseppina Lacerra¹

¹Istituto di Genetica e Biofisica “Adriano Buzzati-Traverso” – CNR, Napoli, Italy, ²Azienda Ospedaliero-Universitaria, Ospedali Riuniti, Foggia, Italy, ³Dipartimento di Biochimica, Biofisica e Patologia Generale, Seconda Università degli Studi di Napoli, Napoli, Italy

Objective and importance: To verify the presence of $\beta$-thalassemia in subjects showing hematologic phenotype of $\alpha$-thalassemia, conduct normal molecular sequence analysis of the $\alpha$-globin genes, and detect the absence of the most frequent $\alpha$-thalassemia deletions.

Clinical presentation: A patient from Apulia (Southern Italy) was referred to our institution for the occasional founding of hypochromic polyglobulia and microcytic red blood cells associated with normal levels of Hb A2 and Hb F and normal iron parameters.

Intervention and technique: The patient has been investigated using Sanger sequencing, multiplex ligation-dependent probe amplification (MLPA), quantitative real-time PCR, restriction analysis, and gap-PCR. A novel deletion, the Italian ($\epsilon\gamma\delta\beta$)$^0$-thalassemia, has been identified. The 5′ breakpoint was within a LINE element of 80 kb 3′ of the $\epsilon$-globin gene, and the 3′ breakpoint was within a 160-bp palindrome of about 30 kb 5′ of the $\beta$-globin gene. The breakpoint region was characterized by the presence of a microhomology (5′-TCT-3′) and of an insertion of 43 bp owing to the duplication of the 160-bp palindrome. Comparison of the Hb and Hb A2 values of ($\epsilon\gamma\delta\beta$)$^0$-thalassemia from the literature with those of (molecularly known) thalassemia carriers indicated a higher level of Hb A2 with respect to $\alpha$-thalassemia and a lower level of Hb with respect to $\beta^0$-thalassemia carriers.

Conclusion: In this study, we report the first ($\epsilon\gamma\delta\beta$)$^0$-thalassemia case identified in Italy. To avoid misdiagnosis of $\beta$-thalassemia, we suggest verifying the presence of large deletions of the $\beta$-globin gene cluster in subjects showing a higher border line level of Hb A2 and a lower level of Hb.

Keywords: ($\epsilon\gamma\delta\beta$)$^0$-Thalassemia, MLPA, novel deletion, Beta-thalassemia misdiagnosis, Italian patient

Introduction

Beta-thalassemia is one of the most frequent hereditary diseases in the Mediterranean area. The quantification of Hb A2 (the minor adult hemoglobin) is essential for the routine diagnosis of beta-thalassemia trait because its concentration is raised in most carriers of beta-thalassemia; moreover, the correct diagnosis is important to prevent the Cooley’s anemia.¹

The ($\epsilon\gamma\delta\beta$)$^0$-thalassemias are rare deletions identified only in heterozygous form. They are caused by long deletions in the $\beta$-globin cluster and molecularly are classified into two groups: group I removes all, or a greater part of the $\beta$-globin cluster, including the $\beta$-globin gene; group II removes extensive upstream regions, leaving the $\beta$-globin gene itself intact although its expression is silenced because of inactivation of the upstream beta-locus control region ($\beta$-LCR).²,³ Up to now, 32 ($\epsilon\gamma\delta\beta$)$^0$-thalassemia deletions have been described, but the complete characterization has been achieved in only 14 of them.³–⁶

Adult heterozygotes show a phenotype similar to that of hematological carriers of beta-thalassemia, but with normal levels of Hb A2 due to the loss of one delta locus and normal or minimally increased Hb F; the red cells are relatively more hypochromic and small. The normal levels of Hb A2 make the hematologic phenotype similar to that of carriers of alpha-thalassemia. As already described, these deletions are characterized by a moderately severe neonatal hemolytic anemia and they are distinguished from beta-thalassemic patients because they may
need red blood cell (RBC) transfusion for the first 6 months of life, but this remits spontaneously with the increasing production of beta-globin in the first year of life.4

Despite the extreme heterogeneity of the molecular basis of the beta-thalassemia in Italy, the \((\epsilon\gamma\delta\beta)^0\)-thalassemia deletions have never been identified.7,8 We carried out an epidemiological project on the molecular basis of alpha-thalassemia in Southern Italy and we identified a patient with hematologic phenotype of alpha-thalassemia (microcytosis, polycythemia, and normal Hb A2), which showed normal molecular sequence analysis of the alpha- and beta-globin genes and absence of the most frequent alpha- or beta-thalassemia deletions. The analysis of the alpha- and beta-globin gene clusters by multiplex ligation probe amplification (MLPA) (MRC Holland) did not reveal the presence of deletions in the alpha-globin genes, but showed the presence of a novel deletion that removes all the beta-globin clusters.9

Patients and methods

Clinical report

The patient was an Italian man, 30 years old, referred to our institution from a local hematological service for the occasional founding of hypochromic polychromatophilia and microcytic RBCs. In the proband, normal levels of Hb A2 (3.1%) and Hb F (1.1%) were associated with normal iron parameters. Other members of the family were not available for the study. The proband was selected by the Thalassemia Center collaborating in this study, among those referred to it for the hematological diagnosis. This study adhered to the tenets of the Declaration of Helsinki. We obtained

Figure 1 Molecular characterization of the Italian \((\epsilon\gamma\delta\beta)^0\)-thal in Italian patient

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B2M: β–pared with a pool of normal subjects (Fig. 1A).

**Hematological data**
Blood counts and Hb analysis were done using standard methods. The hematological data and the serum iron values were determined in the hospitals, by using standard methods. Hb analysis was carried out by cation exchange high-performance liquid chromatography, using the HA-8160 ADAMS (Menarini, Firenze, Italy). The proband was selected on the basis of the normal iron parameters and normal Hb A2 ≥ 2.0–2.2% and ≤3.2–3.4%.

**Molecular analysis**
For the identification of alpha- or beta-thalassemia defects, molecular analyses have been conducted as already reported (see Supplementary Data).

**MLPA and qRT-PCR analysis**
Two MLPA kits (MRC-Holland, Amsterdam, the Netherlands), for the identification of rearrangement in the alpha-globin gene cluster (SALSA MLPA KIT P140 HBA) and in the beta-globin gene cluster (SALSA MLPA KIT P102 HBB), were used according to the manufacturer’s recommendations and as previously reported. MLPA products were separated by ABI-3130XL Genetic Analyzer (Applied Biosystems) with primers chosen outside repeated sequences (Table 1 and Supplementary Data). The beta-2-microglobulin was used as the reference gene.

**Breakpoint characterization**
The genomic DNA encompassing the deletion breakpoint was amplified by long-range gap-PCR using the TripleMasterPCR System (Eppendorf, Hamburg, Germany) with the forward primers p 5'-GCATG TTAGTGGAAATTAGCCA-3' and the reverse primer c 5'-TCAAATGGATCAAGATTTGGA GAGAGT-3', giving rise to a fragment of about 6.5 kb. The region of the breakpoint was reduced by restriction mapping analysis using the enzymes BglII, HindIII, and SacI; new primers were designed (forward primer pl 5'-AGATGGTCTATTTAGGTCCTTGGTGTT-3' and reverse primer cl 5'-CAACTGCTGCTTTTGGTCCA-3') and a new fragment of about 1.7 kb was amplified by long-range gap-PCR and then sequenced (Figs. 1B and 2A).

A gap-PCR for the screening of carriers for the Italian (eγβ)0-thalassemia was set up. The specific deletion amplicon was 1.750 bp (primers cl and pl); the internal control amplicon was 866 bp (forward primers control A 5'-TCAAATGGATCAAGATTTGGA GAGAGT-3' and reverse primers control B 5'-TCACTGCTGCTTTTGGTCCA-3').

**Table 1**

| Name | Fragment length (bp) | Position on chromosome 11 | Forward primer | Reverse primer |
|------|----------------------|---------------------------|----------------|----------------|
| a    | 74                   | 5'-CTAGTGACGGGAGACCTCTGCCTAATGGA-3' | 5'-CTGGGGCATCTCACAATGCTTG-3' |
| b    | 74                   | 5'-AGTGTTAGTGGAAATTAGCCA-3' | 5'-GGCTGCTGAGGTCCTTGGTGTT-3' |
| c    | 74                   | 5'-GGCAATTCCTAGGAGCAAGCCAACCATGCTA-3' | 5'-ATGGCCTAGGGAAGCCCTTAC-3' |
| d    | 74                   | 5'-AAAGGTCTTGACGTTGTG-3' | 5'-TGTCCCCCAAGCCTTATTAATAC-3' |
| e    | 74                   | 5'-TGAGTTCGCTCCTTGGAAGACCAACCATGCTA-3' | 5'-GAGGGAACAGAAGAAATAGGACCATGCT-3' |
| f    | 74                   | 5'-AGCCATCCTGAAATTTTGGC-3' | 5'-GTACACAGAGATGAGACCATCTG-3' |
| g    | 74                   | 5'-CGGTTGCTCTTCTGGTGTCA-3' | 5'-CAGCTTTCTGCTTTTGGT-3' |
| h    | 74                   | 5'-CTCACTGCTAGAACAGCCCAAG-3' | 5'-ACTGCTTCTCCGGTATTGAACCC-3' |
| i    | 74                   | 5'-GCAATTCCTCTCTGCTCTATCAAC-3' | 5'-GCTCCCCCAAGCCTGTTTATAATAC-3' |
| j    | 74                   | 5'-AAATGAAATAGGACAGAGCAAGGCCTCCTG-3' | 5'-TTTGATCTCACAGTGCTGGTCTGT-3' |
| k    | 74                   | 5'-AGCAATGGGCGCCAGCCCTCTCAAATG-3' | 5'-TCTGCAGGTGGACTGAGGATG-3' |
| l    | 74                   | 5'-CTGGAGCAGGCTAGAGAAAGATG-3' | 5'-TCGCTCCCCTCCTTTTGGT-3' |
| m    | 74                   | 5'-CTCTGCTGCTTCTCGAGGATG-3' | 5'-TCCACTGGTTCTGCTGAGGATG-3' |
| n    | 74                   | 5'-TGAGGAGGAGGCTAGAGAAAGATG-3' | 5'-TCGCTCCCCTCCTTTTGGT-3' |
| o    | 74                   | 5'-TGAGGAGGAGGCTAGAGAAAGATG-3' | 5'-TCGCTCCCCTCCTTTTGGT-3' |
| p    | 74                   | 5'-GGTACAGGCTAGGGAAGCCCTTAC-3' | 5'-GTACACAGAGATGAGACCATCTG-3' |
| q    | 74                   | 5'-TCTGCAGGTGGACTGAGGATG-3' | 5'-TCTGCAGGTGGACTGAGGATG-3' |
| r    | 74                   | 5'-AAATGAAATAGGACAGAGCAAGGCCTCCTG-3' | 5'-TCTGCAGGTGGACTGAGGATG-3' |
| s    | 74                   | 5'-AAATGAAATAGGACAGAGCAAGGCCTCCTG-3' | 5'-TCTGCAGGTGGACTGAGGATG-3' |
| B2M  | 80                   | 5'-TCTGCTGCGGAGCTTCTGCTTC-3' | 5'-GGTACAGGCTAGGGAAGCCCTTAC-3' |

Note: Primer positions are according to Reference Sequence GRCh37/hg19 (http://genome.ucsc.edu).

B2M: β2 microglobulin.
Statistical analysis

Statistical analysis was conducted with the software Minitab v.16 using the one-way ANOVA method to compare the hematological data from the carriers of the εγδβ0-thalassemia with that of previously studied α0- and β0-thalassemia carriers. A P-value < 0.05 was considered statistically different.

Database

All the experimental results (hematological, biochemical, and mutations) and the family relationships were collected in anonymous form in a database, developed on Microsoft Visual Fox platform and interfaced with external software such as Microsoft Excel, Microsoft Word, Progeny (Progeny software, LLC, Winsford, UK).

Results and discussion

The proband, showing an alpha-thalassemia phenotype, was enrolled in an epidemiological project on the molecular basis of alpha-thalassemia in Southern Italy (Table 2). The molecular screening for alpha-thalassemia gave negative result: the alpha-globin gene sequencing analysis revealed that the patient was heterozygote for the SNP α2+4 C > G, leading to exclude the presence of deletions involving the alpha-globin genes. We hypothesized the presence of double heterozygosis for the alleles of delta- and
beta-globin, but the beta-globin gene sequencing showed absence of mutation and homozygosis for the beta-globin gene framework.\textsuperscript{3,15}

The MLPA analysis, performed to verify the presence of deletions or rearrangements, showed a normal pattern of the alpha-globin cluster while the analysis of the beta-globin cluster revealed the presence of a long deletion, removing all the beta-globin clusters, including the LCR regions (Fig. 1A). The analysis by qRT-PCR indicated that the deletion starts in a region located about 80 kb 3′ of the epsilon-globin gene, extends for at least 150 kb, and ends about 30 kb 5′ of the beta-globin gene (Fig. 1).

At a first analysis, the deletions looked very similar to the Chilean (εγδβ)\textsuperscript{0}-thalassemia.\textsuperscript{16} By qRT-PCR with primers probing the regions not tested by MLPA, we restricted the possible breakpoint region: the 5′ breakpoint was localized between the qRT-PCR amplicons p and o, and the 3′ breakpoint was localized between the qRT-PCR amplicons d and c. The long-range PCR using the amplicons’ primers c and p (Fig. 1B and Table 2) gave rise to an anomalous fragment of about 6.500 bp; the breakpoint region was further reduced by restriction mapping analysis with the enzymes Bgl II, HindIII, and Sac I. A new fragment of about 1.700 bp was amplified. The Sanger sequence showed that the deletion ranged from position 5,215,626 to position 5,378,422 for a total length of 162,796 bp (Fig. 2B).

The analysis of breakpoints showed that both are within repeated sequencing. In particular, the 5′ breakpoint was in a 5.247-bp LINE element containing the breakpoint of three different deletions: the Norwegian (εγδβ)\textsuperscript{0}-thalassemia, the Dutch III (εγδβ)\textsuperscript{0}-thalassemia, and the English II (εγδβ)\textsuperscript{0}-thalassemia deletions (Fig. 1C).\textsuperscript{17–19} However, the 3′ breakpoint was located within a perfect 160-bp palindrome region that contains the deletion breakpoints of five other deletions: the Chilean (εγδβ)\textsuperscript{0}-thalassemia; the Italian Gγ(λεδβ)\textsuperscript{0}-thalassemia; the Belgian Gγ(λεδβ)\textsuperscript{0}-thalassemia; the Pakistani I (εγδβ)\textsuperscript{0}-thalassemia; and the Indian HPFH\textsuperscript{3,14,16,20,21} All these five deletion breakpoints are very close to the midpoint of the palindrome and localized in a region of 18 bp, as reported in the Italian Gγ(λεδβ)\textsuperscript{0}-thalassemia.\textsuperscript{14} The breakpoint of the Italian (εγδβ)\textsuperscript{0}-thalassemia was localized in the 3′ end of the 160 bp palindrome at position +103 and characterized by the presence of an insertion of 46 nucleotide sequences created by the duplication of the part of the palindrome from position +98 to position +143. A short homology TCT was present in the breakpoint. Palindrome sequences can form stem-loop structures that could favor unequal crossing-over. No direct homology was detected between the 5′ and 3′ normal sequences surrounding the deletion. The exact

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**Table 2** Hematological data of the proband and of the current findings in heterozygotes for the εγδβ\textsuperscript{0}-deletion

| Ref | Sex/age (years) | Case 2 | Case 3 |
|-----|-----------------|--------|--------|
| RBC (10\textsuperscript{12}/L) | 5.25 | 5.1 | 5.47 |
| Hb (g/dL) | 11.8 | 11.2 | 11.2 |
| MCV (fL) | 74.3 | 53.0 | 50.9 |
| MCH (pg) | 23.9 | 29.0 | 27.1 |
| MCHC (g/dL) | 31.1 | 31.1 | 31.1 |
| Serum iron (μg/dL) | 89 | 39.6 | 39.6 |
| Hb A2 (%) | 3.1 | 2.9 | 3.0 |
| Hb F (%) | 0.1 | 0.3 | 0.1 |

Ref: references; ad: adult.
mechanism causing the breakage event remains speculative, but the cluster of deletion breakpoints suggests that the two regions (LINE and 160-bp palindrome) are highly prone to recombination. In addition, the presence of a short homology (5′-TCT-3′) could favor the non-homologous recombination.22,23

Complete characterization of deletion is important for genetic counseling and may lead to delineation of novel regulative regions. In the present case, we showed that the definition of the breakpoints of deletion is possible also in the presence of repeats although technically challenging.

The new deletion removes all the genes of the beta-globin cluster and it was for the first time identified in an Italian patient and therefore has been called Italian (εγδβ)0-thalassemia. The Hugo nomenclature was NC_000011.10:g.[5194397_5357192del;5194356_5194401insAGCTAAAGGTTTTGTAAATGCACCAA TCAGCAATCTGTGTCCTAACTC]. Although these kinds of deletions affected a moderately severe neonatal hemolytic anemia, our patient did not show any such occurrence.

Several deletions (β0)-thalassemia, (δβ0)-thalassemia (Sicilian, Spanish, Turkish/Macedonian), and (γδβ)0-thalassemia deletions (Turkish, Italian) have been described in Italy and in the Mediterranean area. All of them are characterized by microcytosis, normal Hb A2, and increased Hb F level, with the last marker indicating the possible presence of a deletion of the beta-globin gene cluster.24–27 Conversely, the carrier of the Italian (εγδβ)0-thalassemia shows a beta-thalassemia phenotype with normal Hb A2 and Hb F level.

Analyzing the phenotype of the new deletion, we were intrigued by the Hb A2 (3.1%) that is borderline and by the low hemoglobin level (11.8 g/dL). To test the significance of these values, we looked for the phenotype of the (εγδβ)0-thalassemia carriers from literature (Table 2).3–6,16–19,28–30 We compared (i) the Hb A2 values of the 19 (εγδβ)0-thalassemia carriers

![Figure 3](image-url)
carriers with those of 92 males and females, selected from our database, heterozygotes for an α-thalassemia deletion [-α]20.5, -MED, -CAL and showing normal iron parameters; i) the Hb levels of the (εγδβ)0-thalassemia carriers with those of the 92 α-thalassemia and 291 β-thalassemia carriers, with known genotype, selected from our database. The Hb A2 level of the (εγδβ)0-thalassemia carriers was of 2.9 ± 0.34 (range 2.4–3.8), while the Hb A2 level of the α-thalassemia carriers was of 2.39 ± 0.25 (range 2.0–2.9), with Student’s t-test indicating a significance at >0.01. The Hb level of the (εγδβ)0-thalassemia showed a mean of 10.18 ± 1.56 (range 6.8–13.2) significantly lower than that of the β0-thalassemia carriers 12.16 ± 1.22 (range 9.5–16.0) or α0-thalassemia carriers 12.88 ± 1.19 (range 10.4–16.4), with Student’s t-test indicating a significance at >0.01. These Hb values are similar to those observed in thalassemia intermediate patients (Fig. 3).

In conclusion, the beta-thalassemia with normal Hb A2 could be due to the presence of double heterozygosis for the alleles of delta- and beta-globin genes, as we already reported in 18 unrelated families, or due to the presence of long deletions involving all the beta-globin gene clusters. In the first case, the segregation patterns and the sequencing analysis of the beta- and delta-globin genes are of great help. In case of (εγδβ)0-thalassemia heterozygous, we showed that the Hb A2 value is expected to be a little bit higher than in case of α0-thalassemia carriers and the Hb level is expected to be lower than β0-thalassemia carriers. These two data could favor a differential diagnosis of (εγδβ)0-thalassemia (Table 2 and Fig. 3).

For providing genetic counseling and to prevent misdiagnosis of beta-thalassemia, further to the first identification of an Italian (εγδβ)0-thalassemia, we suggest that MLPA analysis (for the identification of beta-globin gene cluster deletions/duplication) or screening for the Italian (εγδβ)0-thalassemia by gap-PCR should be carried out in patients showing alpha-thalassemia phenotype, but detection of the absence of common or rare alpha-thalassemia deletions and perhaps normal beta-globin gene sequence, in order to ascertain the presence of deletions causing (εγδβ)0-thalassemia. In particular, the search for deletions should be tested in carriers analyzed in the past showing a biosynthetic ratio of beta-thalassemia but the absence of point mutations or deletion, in subjects showing thalassemia, low level of Hb, and border line level of Hb A2 but the absence of the frequent mutations/delitions in the beta- or alpha-globin gene cluster. In our experience, the first identification of deletions and the screening could allow subsequent identification, as reported in the case of the Italian Gγ(εγδβ)0-thal deletion and of the (αα)5.3.

Acknowledgments
We would like to thank the patients for their participation and cooperation.

Disclaimer statements
Contributors Conceived and designed the experiments: G.L., F.D.B., G.L. Analyzed the data: G.L. Contributed reagents/materials/analysis tools: F.D.B., C.S., R.P., G.L. Wrote the paper: G.L. Revised the article critically: C.S. Selected the patients and contributed clinical data: S.D., C.S.

Conflict of interest The authors declare that they have no competing interest.

Funding This research was supported by a grant from Ministero Istruzione, Università e Ricerca (MIUR), Legge 488/92 (Cluster C02, Project 2).

Ethics approval This study adhered to the tenets of the Declaration of Helsinki. We obtained written informed consent from participant for the use of blood sample. This study was also approved by the institutional ethical committee.

ORCID
Giuseppina Lacerra http://orcid.org/0000-0003-1038-8363

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