CASE REPORT

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The need to perform α-thalassemia genetic testing in Italian patients with β-thalassemia trait: A case report

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Abstract
Here, we describe a case report of a Sardinian woman diagnosed as pure beta-thalassemia carrier for her anemia who underwent to alpha-thalassemia genetic testing that revealed she was heterozygous for both thalssemias. This allowed to reach a conclusive diagnosis useful for family counseling and for assess the reproductive risk.

KEYWORDS
alpha-thalassemia, beta-thalassemia, gap-PCR, genetic counseling, MLPA

1 | INTRODUCTION

Italy is historically a natural reservoir of hemoglobinopathies, especially α- and β-thalassemia, that are endemic in the South of Italy, in Sicily and Sardinia. In the last decades, due to internal migrations and immigrants coming from countries where hemoglobinopathies incidence is high, the frequency of hemoglobin variants raised also in other Italian regions.

Alpha-thalassemia is caused by autosomal recessive mutations in *HBA1* and *HBA2* genes encoding, respectively, for α1-globin and α2-globin. Most of mutations (~85%) are due to common deletions involving one or both α-globin genes on one or both alleles. The phenotype of patients depends on the number of α-globin genes involved: dysfunction or absence of all four α-globin genes is associated with hemoglobin Bart hydrops fetalis (Hb Bart), the most severe form of alpha-thalassemia, with intrauterine or neonatal death. When three α-globin genes are involved, patients show hemoglobin H disease (HbH), a severe condition characterized by microcytic hypochromic hemolytic anemia, splenomegaly, and jaundice.

Deletions or dysfunction of two α-globin genes lead to alpha-thalassemia trait while one dysfunctional gene is linked to silent carrier, often asymptomatic.[1]

Alpha-thalassemia trait shares some hematological features with β-thalassemia trait. In some geographic areas of Italy where malaria was endemic, both α- and β-thalassemia are often coinherited, but α-thalassemia is generally underdiagnosed and double heterozygotes for both α- and β-thalassemia are usually diagnosed as pure β-thalassemia carrier, leading to an incomplete diagnosis and, depending on the alpha-globin cluster genetic trait of the partner, offspring risk to have HbH disease.[2]

2 | CASE REPORT

Here, we describe a Sardinian 33 y.o. woman with diagnosis of hypochromic microcytic anemia apparently with no family history of thalassemias, born from healthy non-consanguineous parents and with two healthy sisters. Patient's geneticist suggested to investigate the genetic cause of her anemia to evaluate the offspring risk.

Red blood cell (RBC) indices, part of the complete blood count (CBC) test, revealed: higher red blood cell count (RBC) value (5.44×10^{12}/L; Reference Value (RV): 4.00–5.00×10^{12}/L), microcytosis due to lower mean corpuscular volume (MCV) value (76.1 fl; RV: 80.0–99.0 fl) and hypochromia due to lower mean corpuscular hemoglobin (MCH) value (24.1 pg; RV: 26.0–36.0 pg). The other RBC indices were normal: hemoglobin (Hb) value was 131.0 g/L (RV: 120.0–160.0 g/L), hematocrit (Ht) value was 42.4% (RV: 36.0%–46.0%), mean corpuscular hemoglobin concentration (MCHC) was 316.0 g/L (RV: 310.0–360.0 g/L), and red cell distribution (RDW) value was 13.6% (RV: 11.0%–14.0%). All the reference values reported referred to age and sex of the patient.

Hemoglobin electrophoresis was performed and showed the presence of high level of HbA2 fraction (5.4%) that is the standard diagnostic marker of β-thalassemia. Genetic testing for β-thalassemia revealed the most common Sardinian mutation, the *HBB* c.118C>T (also known as β°39 variant) in heterozygosity. Segregation study was performed and discovered the presence of the c.118C>T pathogenic variant in the patient's father. Based on this result, the patient received the diagnosis of β-thalassemia carrier.

Because of high prevalence of α-thalassemia in some Italian regions and considering the hematological features, under patient's geneticist suggestion, we also performed α-thalassemia genetic test. In our laboratory, Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA) are performed to achieve a detection rate of >99%. Sanger sequencing was performed using non-specific forward primer (5′-CGCCCCAAGCATAAACCCTG-3′) for both *HBA1* and *HBA2* genes, while reverse primers are designed using the 3′UTR specific sequence of the two genes, respectively, 5′-GGGGGGAGGCCCAAGGGGCAAGAA-3′ for *HBA1* and 5′-GGGAGGCCCAGCGGGCAGGAGGAAC-3′ for *HBA2*, using the following amplification conditions: 95°C for 10 min; 95°C for 30 s, 64°C for 30 s, 72°C for 30 s, 40 cycles; and 72°C for 10 min. Electrophoresis analysis was performed and the purified electrophoresis products then sent for Sanger sequencing. Then, sequencing reactions were performed with ABI 3500XL Genetic Analyzer. The sequenced data were analyzed with Mutation Surveyor® (SoftGenetics) software using GenBank NG_000006.1 as their reference sequences.

It revealed no mutations in *HBA1* gene while *HBA2* gene was not analyzed because no PCR product was present after amplification (data not shown). MLPA analysis using SALSA® MLPA® Probemix P140-C1 HBA (MRC-Holland) showed a heterozygous deletion involving part of the alpha-globin cluster, with absence of probes on *HBA2* gene (Figure 1B). For interpretation of results, we used MLPA tables that show expected probe ratios for a selected subset of copy number changes and polymorphisms, for common and uncommon alpha-globin gene cluster mutations. For this case, table of expected probe ratios for common *HBA* deletion types was selected:[3] value of ratio equal to 1 indicates the presence of both alleles, a ratio of 0.5 the loss of one allele and 0 the loss of that probe in both alleles. Three probes are non-specific and detect sequences present in both *HBA1* and *HBA2* genes. In these cases, a ratio of 1 indicates the presence of the two genes in both alleles, a ratio of 0.75 the presence of one gene in both allele and one gene only in one allele, a ratio of 0.5 can represent or the presence of the two genes in *cis*, two genes on only one allele and the deletion of both genes on the other allele (α°), or the presence of the two genes in *trans*, one on an allele and one on the other allele (α⁺). A ratio of 0.25 is linked to the presence of only one gene on one allele and the deletion of the other gene for both alleles while a ratio of 0 indicates the deletion of both genes in both alleles. Considering proband values of ratio, we supposed the presence of the two of the most common α-thalassemia deletions in *HBA2* gene, the −3.7 kb on one allele and the −4.2 kb deletion on the other allele. Segregation study on the patient's parents was performed and showed that her mother carried the −3.7 kb deletion while patient's father was compound heterozygous for both deletions as the proband. We could not analyze the two sisters (Figure 1A). Multiplex Gap-polymerase chain reaction (Multiplex Gap-PCR), the most common method used to screen for common deletions causing α-thalassemia,[4] confirmed the presence of these deletions, showing that the proband and her father present the two common deletions, the −3.7 kb on one allele and the −4.2 kb deletion on the other allele ($-\alpha^{3.7}/-\alpha^{4.2}$), which are rarely reported in combination, while the proband mother was heterozygous for the −3.7 kb deletion ($-\alpha^{3.7}/\alpha\alpha$) (Figure 1C).

3 | DISCUSSION

In our proband, that moved from Sardinia to Lombardy, with a diagnosis of β-thalassemia carrier, genetic testing for α-thalassemia revealed the coinheritance of both α- and β-thalassemia traits. Genetic testing for both thalassemias is recommended in areas where malaria was endemic, as in some regions of Italy like Sardinia, but also in regions of Italy where internal immigrations from regions with high incidence of thalassemias or immigrations from developing countries where hemoglobinopathies are more frequent, particularly from Mediterranean area, Indian Sub-Continent and South-East Asia, were increased in the last decades, like Lombardy. This could be useful not only for prenatal screening program[2] or to make a differential

FIGURE 1 (A) Pedigree of the family. (B) Graph showing the results for the proband generated by Coffalyser.Net software. The x-axis represents the probes (name and length) and the y-axis the ratio of intensity between the samples and the mean intensity of reference samples. (C) Multiplex Gap-PCR results on 1% agarose gel. The amplicon sizes for each deletion are indicated with arrows: the 3.7 kb deletion produces an amplification product of 2022 bp size while the 4.2 kb deletion a product with a size of 1628 bp. F, Father; M, Marker; Mo, Mother; P, Proband; W, Water.
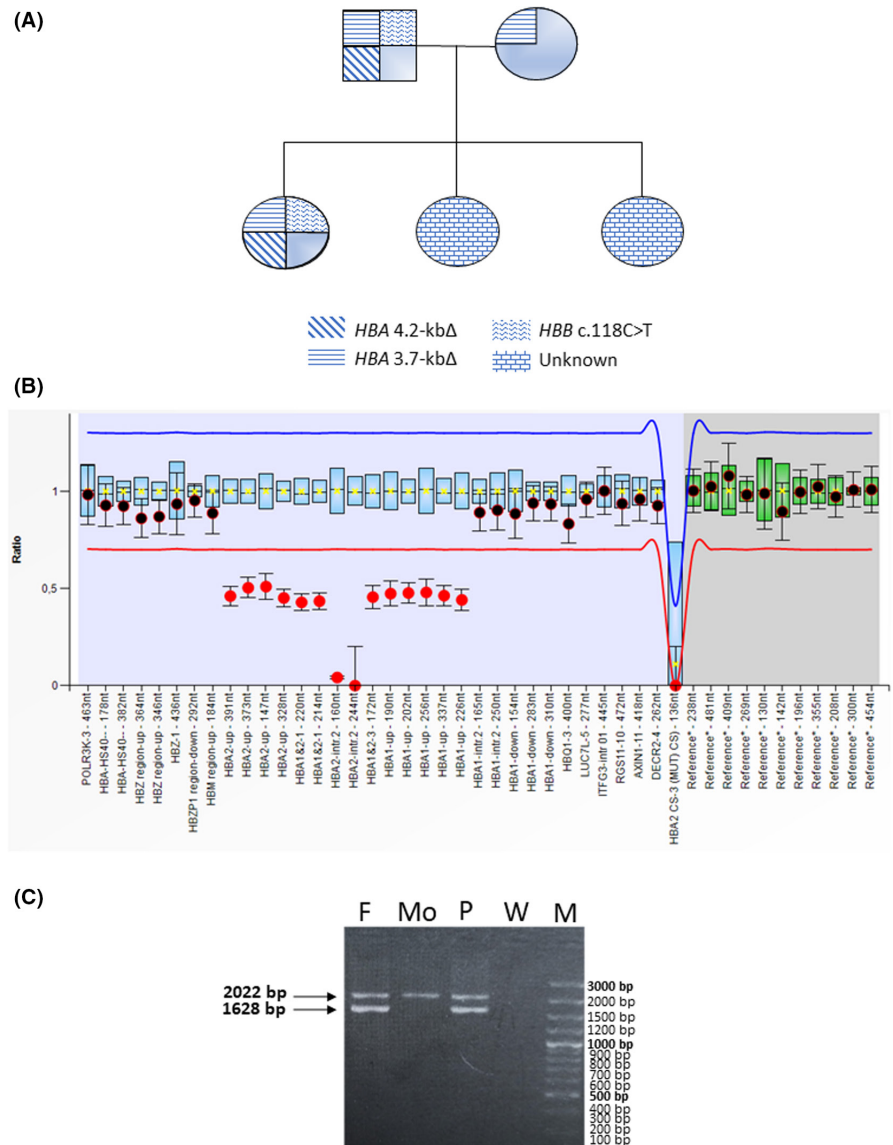
diagnosis with thalassemia due to iron defiency,[5] but also for family counseling, to assess β-thalassemia risk and to avoid the potential risk of HbH disease in case of marriage between the proband, α^+ carrier, with a α° partner.

Even if α-thalassemia is one of the most common genetic disorders affecting the 5% of world's population,[6] ß-thalassemia is the most common autosomal recessive disorder in Sardinia, where approximately 10.3% of the population are carriers.[7] The high prevalence of ß-thalassemia carriers leads to an underdiagnosis of α-thalassemia: hematological features of β-thalassemia carrier are the similar to α-thalassemia carrier and the presence of higher percentage of HbA2 is often sufficient to diagnose β-thalassemia, without performing further analysis to investigate about α-thalassemia. In this study, the proband seems to match β-thalassemia carrier features, but considering her Sardinian origin, both α- and β-thalassemia have been investigated. We found that she

is a double heterozygote for both thalassemias, in particular she is a compound heterozygous for α-thalassemia, carrying the common deletions $-\alpha^{3.7}/-\alpha^{4.2}$, and a heterozygote for β-thalassemia carrying the pathogenic variant c.118C>T (ß°39) on *HBB* gene. Individuals who co-inherit α- and β-thalassemia have impaired synthesis of both chains, displaying an improved phenotype due less unbalanced globin chain synthesis.[2]

Diagnosis of α-thalassemia requires a combination of several laboratory tests combined with hematological analyses and genetic testing. There are several molecular techniques used for α-thalassemia genetic testing: Multiplex Gap-PCR, Loop-mediated isothermal amplification (LAMP), MLPA, and single tube multiplex PCR, are used to detect α-thalassemia common deletions while Sanger sequencing is a commonly used best technique for thalassemia diagnosis even if this method cannot detect deletion mutation, which is the common cause of

α-thalassemia. Recently, Next-Generation Sequencing (NGS) has been introduced to overcome these technical issues even if the complexity of the alpha-globin cluster gene and the high cost have not allowed yet to introduce it as a routine test for α-thalassemia. On the contrary, NGS could be a useful tool for both thalassemias screening program, especially in these cases of coinheritance, because a targeted gene sequencing panel with all genes involved in alpha- and beta-globin cluster could be analyzed at the same time and more patients' samples could be loaded in a single experiment. This could lead to conclude the genetic diagnosis with a single test reducing the turn around time (TAT) and optimizing the cost. Therefore, the choice of the appropriate genetic testing depends on laboratory's equipment, know-how and cost involved.[8]

In our laboratory, we performed sanger sequencing for single nucleotide variants (SNVs) detection and MLPA technique for copy number variants (CNVs). To date, few studies about the utility of MLPA in routine clinical laboratory settings have been conducted.[9,10] MLPA could play an important role in the diagnosis because it allows to analyze in a single experiment a wide range of mutations affecting the α-globin cluster gene, not detected by other techniques. In areas where both alpha- and beta-thalassemia are present, MLPA is recommended as first tier screening to detect the wider range of mutations in the α-globin cluster gene. Meanwhile, Gap-PCR is the most used method to screen for common deletions, but it is not useful for new or rare deletions. In this study, we used Gap-PCR only to confirm deletions detected using MLPA. Finally, segregation study was mandatory to better characterize the pathogenic variants detected and to define the conclusive genotype of the patient.

4 | CONCLUSION

This case report outlines the importance of α-thalassemia genetic testing in β-thalassemia carriers in Italy both in regions where α-thalassemia and β-thalassemia genes are often mutated and in regions where immigrations of some ethnic group from developing countries, where these conditions are endemic, increased in the last decades. We suggest performing both Sanger sequencing and MLPA technique to detect a wider range of mutations causing α-thalassemia. However, the rapid evolution and widespread use of NGS in clinical laboratories and the reduction in costs of sequencing reactions could enable to NGS to be considered in the next future as valid screening method, especially in Italian regions with high prevalence of thalassemias. Our group is working to introduce as soon as possible this method we already use for other genetic disease like cystic fibrosis also for thalassemias screening program. That could be useful for a more accurate diagnosis and better family counseling to assess the reproductive risk of both alpha- and β-thalassemia, avoiding incomplete diagnosis that could lead to severe α-thalassemia.

CONFLICT OF INTEREST
The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT
Raw data were generated in the laboratory of Synlab Italy (Castenedolo, BS, Italy). The data derived supporting the findings of this study are available from the corresponding author upon request.

CONSENT
Published with written consent of the patient and her parents.

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