

Hematologic and molecular characterization of a Sicilian cohort of  $\alpha$  thalassemia carriers

Detailed hematologic and molecular analyses were carried out on a cohort of Sicilian individuals with suspected or asymptomatic  $\alpha$ -thalassemia. Iron deficiency, mild  $\beta$ -thalassemia alleles and most common Mediterranean  $\alpha$ -globin deletional mutations were excluded. All negative individuals were then tested for  $\alpha$ -thalassemia point mutations by a denaturing high-performance liquid chromatography (DHPLC)-based assay. Four rare  $\alpha$ -globin variants (Hb Interlaken, Hb Chesapeake, Hb Lombard, Hb Sun Prairie) and one point mutation (polyA: AATAAA-G in  $\alpha 2$ ) were identified in 15 out of 80 carriers. Direct sequence analysis carried out in the remaining 65 negative individuals revealed no further sequence variants.

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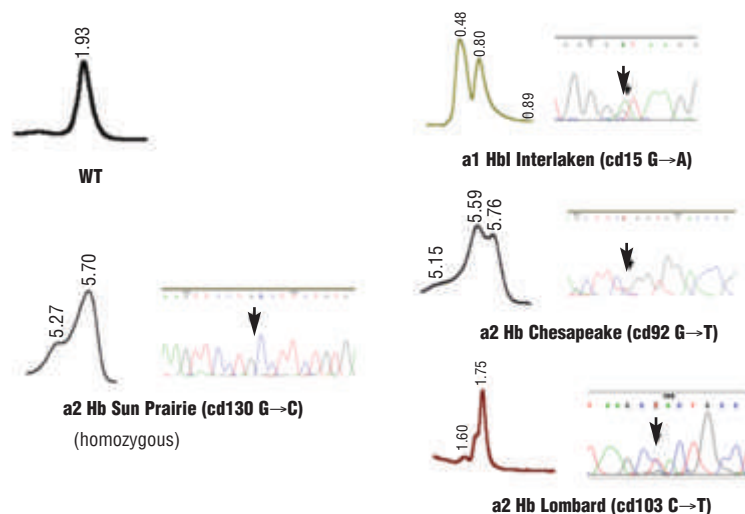
$\alpha$ -thalassemias are hematologic diseases arising from more than 80 different genetic alterations, affecting one or both copies of the duplicated  $\alpha$ -globin genes ( $\alpha 1$  and  $\alpha 2$ ) located in 16p13.3.<sup>1</sup> Although most causative alterations are large genomic deletions, at least 48 non-deletion point mutations have also been reported so far.<sup>2</sup> The clinical severity of the disease is strictly related to the number of altered genes, ranging from the fatal Hb Bart's hydrops fetalis syndrome (four affected genes), to the moderately severe HbH disease (three affected genes), to the mild microcytic, hypochromic anemia (two affected genes), and to the clinically silent or asymptomatic form (one affected gene).<sup>3</sup> The diagnosis of the most severe forms of  $\alpha$ -thalassemia is primarily based on routine hematologic and biochemical analyses, while the clear

identification of healthy carriers and/or  $\alpha$ -thalassemia patients presenting a moderate or silent phenotype is often hindered by the absence of specific diagnostic hematologic parameters.<sup>4</sup> In these latter cases, molecular DNA analysis becomes a fundamental tool to validate hematologic findings, especially when other possible explanations for microcytosis or mild anemia (such as rare  $\beta$ -globin mutations or iron deficiency) have been ruled out.

In this study, 83 individuals of Sicilian origin with mild anemia and hematologic parameters (mean corpuscular volume <78 fL; mean cell hemoglobin <27 pg; HbA2<3.3%) suggestive of possible carriership of a silent form of  $\alpha$ -thalassemia were selected for molecular screening (Table 1). The study was conducted in accordance with the Declaration of Helsinki and blood samples were obtained after informed consent had been given. All 83 subjects were negative for structural hemoglobin variants (including HbH) and only three of them had low serum ferritin levels (6.2; 6.7 and 10.5 ng/mL). These three individuals were excluded from further molecular analyses. To rule out the occurrence of normal HbA<sub>2</sub>  $\beta$ -thalassemia in the remaining 80 subjects, rare or silent  $\beta$ -thalassemia alleles were excluded by direct sequencing of the full genomic  $\beta$ -globin region. The most common Mediterranean  $\alpha$ -globin deletional ( $-\alpha^{37}$ ;  $-\alpha^{42}$ ;  $-\alpha^{20.5}$ ;  $-\alpha^{Med}$ ;  $-\alpha^{Cal}$ ) and point mutations or microdeletions ( $\alpha^{Hiph1}$ ;  $\alpha^{Ncol}$ ;  $\alpha\alpha^{Ncol}$ ) were also excluded by GAP-polymerase chain reaction (PCR) and enzymatic restriction assay, respectively.<sup>4,5</sup> All these individuals were then analyzed for the presence of rare point mutations or micro-deletions/insertions in both  $\alpha$ -globin genes by a previously described denaturing high performance liquid chromatography (DHPLC)-based assay.<sup>6</sup> A semi-nested PCR with an initial amplification step using one  $\alpha$ -globin gene specific primer, located at the divergent 3' end, was carried out to discriminate between mutations located in  $\alpha 1$  or  $\alpha 2$ -globin genes.<sup>7</sup> After DHPLC analysis and subsequent direct DNA sequencing of altered fragments, four known structural  $\alpha$ -globin variants were detected in one

Table 1. Hematologic parameters and diagnosis in the 83 Sicilian individuals investigated for suspected  $\alpha$ -thalassemia.

No. of subjects in final diagnosis group	Mean Hb (g/dL)	Mean MCV (fL)	Mean MCH (pg)	Mean HbA2 (%)	Mean ferritin (ng/mL)	Mean transferrin (mg/100mL)	Mean serum ferritin ( $\mu$ g/100 mL)	$\alpha$ -globin mutation
3	10.96 $\pm$ 1.26	74.22 $\pm$ 3.08	24.44 $\pm$ 1.48	2.26 $\pm$ 0.68	7.96 $\pm$ 2.63	286.66 $\pm$ 58.04	64.00 $\pm$ 16.37	Iron deficiency
1	8.8	71.06	22.74	1.5	21.7	188	99	Hb Sun Prairie (homozygous)
1	11.6	70.92	23.11	2.9	54	231	158	Hb Interlaken
1	15.5	75.00	24.22	3.3	63.4	291	98	Hb Chesapeake
1	13.4	79.60	24.63	2.7	57.9	238	94	Hb Lombard
11	12.76 $\pm$ 1.09	74.75 $\pm$ 3.01	24.80 $\pm$ 0.86	2.6 $\pm$ 0.19	41.77 $\pm$ 22.84	286.11 $\pm$ 49.82	78.18 $\pm$ 13.77	Poly A (AATAAA-G)
65	12.17 $\pm$ 1.44	71.31 $\pm$ 10.08	21.09 $\pm$ 2.60	2.45 $\pm$ 0.50	76.30 $\pm$ 179.85	293.70 $\pm$ 63.44	74.98 $\pm$ 31.31	No mutation



**Figure 1.** DHPLC chromatograms and sequence analysis of the four rare  $\alpha$ -thalassemia mutations detected in this study.

individual each: Hb Interlaken, Hb Chesapeake, Hb Lombard, and Hb Sun Prairie (Figure 1). One common mutation affecting the polyA sequence of the  $\alpha 2$  globin gene (polyA: AATAAA-G) was detected in 11 heterozygotes, and a common neutral polymorphism (+861 G-A in  $\alpha 2$ ) was detected in 26 individuals. While Hb Lombard is typical of the Italian population and the Hb Interlaken variant has already been described in Italy, Hb Chesapeake has been found in a German and in a Japanese family, the poly-A mutation is common among Arabs and the Hb Sun Prairie has been found only twice in young men of Indian descent.

In addition to known causative alterations, two novel sequence variations in intronic regions (IVS1-27 C-T in  $\alpha 2$  and IVS2-122 G-T in  $\alpha 1$ ) were identified in four and three individuals, respectively; and two different point substitutions were detected in the 3' UTR of the  $\alpha 1$  globin gene (3'UTR Term+46 C-A and 3'UTR Term+98 C-G) in one and four individuals, respectively. Although these previously unreported sequence changes were absent in 90 normal controls, they must be considered neutral, unless functional data are provided to support their definitive role in the pathogenesis of  $\alpha$ -thalassemia. In spite of direct sequencing analysis performed on the full genomic region of the two  $\alpha$ -globin genes, no pathogenic mutations were detected in the remaining 65 individuals, thus confirming a 100% concordance with the DHPLC results.<sup>6,8</sup> It is likely that the causative genetic alterations in these cases are located in  $\alpha$ -globin regulatory regions (e.g. the HS-40) or in different modifier genes.<sup>9</sup> Overall, the DHPLC analysis was carried out in less than a week and was found to be highly reproducible. This approach could be useful for molecular diagnosis of both silent and mild  $\alpha$ -thalassemia carriers when common deletional mutations have been excluded and when a preliminary screening method is needed to select a mutated fragment for sequencing, with consistent reductions in the time and cost of analysis.

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