Genotyping for known Mediterranean \(\alpha\)-thalassemia point mutations using a multiplex amplification refractory mutation system

We report the conditions of a multiplex-amplification refractory mutation system (ARMS) for genotyping for nine known mutations of the \(\alpha\)-2-globin gene and of the ARMS assay for the detection of \(\alpha\)-1 Hb J-Oxford and \(-\alpha3.7-\)AC. The method is reproducible, reliable, simple, rapid, inexpensive and provides genotype diagnosis in >70% of point-mutation carriers in Mediterranean countries. Moreover, it allows investigation of the structure of mutated alleles by sequencing ARMS-amplicons.

\(\alpha\)-thalassemia is a hereditary microcytic anemia caused by structural defects involving one or both of the duplicated 5’-3’ \(\alpha\)2 (833 bp) and \(\alpha\)1 (841 bp) globin genes, clustered in tandem on chromosome 16 and showing >96% homology.\(^1\)\(^2\) The hematologic diagnosis is hindered by the absence of markers. Achieving a molecular diagnosis is relevant to the prevention of severe \(\alpha\)- and \(\beta\)-thalassemia. A large spectrum of deletions and point-mutations have been reported in the Mediterranean area.\(^3\) A certain number of polymerase chain reaction (PCR)-based methods have been set up to detect point-mutations; most of these require time-consuming and expensive analyses of the amplicons.\(^3\) Amplification refractory mutation system (ARMS) is a well-known method based on the synthesis of mutation-length-specific amplicons, identifiable with agarose-gel electrophoresis.\(^4\) A multiplex ARMS has been optimized for the detection of six point mutations common in South East Asia.\(^5\) We established the ARMS-PCR conditions for genotyping for known Mediterranean \(\alpha\)-globin mutations by carrying out a retrospective study of previously reported individuals 36 carriers, three homozygotes, four compound heterozygotes (deletion/point-mutation genotype) and ten normal subjects.\(^2\) The primers and specific PCR conditions used are described in Table 1 (online version only); other PCR details were as previously reported.\(^5\) We developed two approaches. The first used multiplex-ARMS to screen for nine known mutations of the \(\alpha\)-2-

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**Figure 1.** Separation of allele-specific amplicons on agarose gel electrophoresis. A. Multiplex-ARMS assay for the point mutations of the \(\alpha\)-2-globin gene; B. ARMS with the normal primers corresponding to the mutations reported under each lane; C. ARMS for the mutation \(\alpha\)-1 cod 15 GTA→ATA (Hb J-Oxford); D. ARMS for the mutation \(-\alpha\)3 17 +35/36 –AC. In the upper part of each electrophoretic record there is the design of the functional structure of the gene (three exons and two introns, 5’ and 3’ UNT regions); the small triangle represents a 7-bp insertion in the IVS-II of the \(\alpha\)-1-globin gene. The positions of the ARMS-primers are marked with an asterisk, that of the common primers with an arrow. Genotypes of the analyzed subjects are indicated under each lane. The symbol of a single mutation indicates heterozygotes; hom+mutation indicates homozygotes; mutation/deletion indicates compound heterozygotes. Control-amplicons of 714 bp (A, B, D) or of 346 bp (C) were amplified. A 50 bp ladder was used as the length marker.
globin gene and to define the relative heterozygous/homozygous state (Table 1, A-B, online version only). The carriers were first analyzed with the multiplex-assay for the three most frequent mutations in our area (α2-ATG→ACG, α2-IVS-1 -5nt, α2-AATAAA→AATAAG) plus Hb Icaria; negative subjects were screened for five mutations (α2-ATG→GTG, Hb Caserta, Hb Sun Prairie, Hb Constant Spring, α2-AATAAA→AATGAA) showing lower relative frequencies. These two multiplex-steps were necessary because mutations at the same site produce amplicons with the same length (Table 1, A). Positive subjects were analyzed with an ARMS-assay for the detection of corresponding wild-type sequences by using normal ARMS-primers; assay-conditions were set up for seven of the nine mutations because homozygous or deletion/point-mutation compound heterozygotes for the remaining two were not available (Table 1, B).

The second approach was directed to detecting of specific mutations of the α1-globin gene (Hb J-Oxford) or of the αα3.7 hybrid-gene (+35/36 -AC) with an ARMS assay (Table 1, C); it was performed in carriers of Hb J-Oxford-like variant or of the hybrid-gene. All the amplicons synthesized with different approaches were separated by 1.5% agarose-gel electrophoresis. The expected mutation-length-specific ARMS-amplicons (Table 1) were detected in heterozygotes (Figure 1, A; C-D), homozygotes and compound heterozygotes. The expected amplicons synthesized with normal ARMS-primers were detected only in normal subjects and carriers and never in homozygotes or deletion/point-mutation compound heterozygotes (Figure 1, B). The only exception was that carriers of Hb Caserta (n=1) and Hb Sun Prairie (n=2) showed both the ARMS-amplicons expected for the two mutations (Figure 1, A). In order to highlight the association in cis of these two mutations and the structure of the other mutated alleles, we sequenced all mutation-specific-amplicons. Hb Sun Prairie was found in cis with Hb Caserta and with the neutral SNP +861 G→A; thus, this allele most probably had a different origin from another Hb Sun Prairie associated in cis with the mutation αα3.7 C→T. The αα3.7 associated with +35/36 -AC was found to be of type-II, as already described in Mediterranean families. Hb Constant Spring was confirmed to be associated in cis with the neutral SNP α2 +861 G→A.

In order to validate the multiplex-ARMS, 50 normal subjects from unrelated families were screened. These subjects had a mean corpuscular volume ±78fL, a mean cell hemoglobin ±27pg, normal serum-iron and did not carry deletions or variants detectable by high performance liquid chromatography. Informed consent was obtained from all subjects. Thirty-five subjects were carry deletions or variants detectable by high performance liquid chromatography (in heterozygotes) associated with DNA sequencing of both α-globin genes (in negative subjects) confirmed results and showed that out of the remaining 15 subjects, 4 had rare mutations and 11 had no sequence variations. Out of these, two had most probably had mild sideropenia.

The multiplex-ARMS and other ARMS-assays we set up gave reproducible and 100% reliable results. The method is simple and rapid and has a reasonable cost. It is most important to point out that, out of the α2 point mutations included in the multiplex-ARMS, three (ATG→ACG; IVS-1 -5nt, AATAAA→AATAAG) account for >70% of all point mutations in the Mediterranean area, one (Hb Constant Spring) is among the most frequent in Asiatic immigrants. Thus, in Mediterranean countries the multiplex-ARMS could be of great help as a rapid screening method, for genotype definition in doubtful cases and for confirmation of mutations detected with indirect methods. Moreover, it could be of considerable aid in the analysis of mutated-allele structure for correct definition of genotypes without family studies.

Giuseppina Lacerra, Gemma Musolino, Francesca Di Noce, Romeo Prezioso, Clementina Carestia  
Istituto di Genetica e Biofisica “Adriano Buzzato-Traverso”,  
CNR, Naples, Italy

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E-mail: carestia@igb.cnr.it

References