Genotyping for known Mediterranean α-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 α2-globin gene and of the ARMS assay for the detection of α1 Hb J-Oxford and -α3.7 -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.

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α-thalassemia is a hereditary microcytic anemia caused by structural defects involving one or both of the duplicated 5'-3' α2 (833 bp) and α1 (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 α- and β-thalassemia. A large spectrum of deletions and point-mutations have been reported in the Mediterranean area.[1] 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.[3] A multiplex ARMS has been optimized for the detection of six point mutations common in South East Asia.[4] We established the ARMS-PCR conditions for genotyping for known Mediterranean α-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.[5] 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 α2-

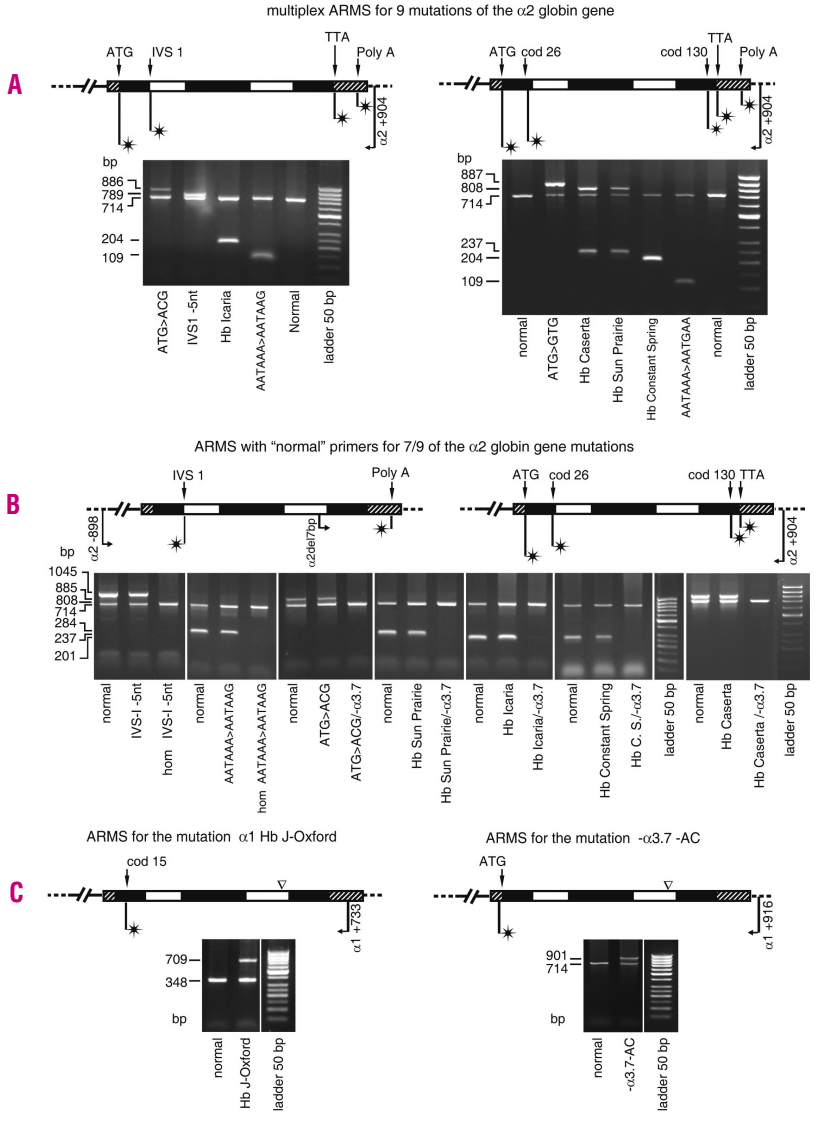

Figure 1. Separation of allele-specific amplicons on agarose gel electrophoresis. **A.** Multiplex-ARMS assay for the point mutations of the α2-globin gene; **B.** ARMS with the *normal* primers corresponding to the mutations reported under each lane; **C.** ARMS for the mutation α1 cod 15 GTA→ATA (Hb J-Oxford); **D.** ARMS for the mutation -$\alpha^{3.7}$ +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 α1-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 348 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-I -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 homozygotes 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 –α3.7 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).[1] 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 α+36 C→T.[6] The –α3.7 associated with +35/36 –AC was found to be of type-II, as already described in Mediterranean families.[7] Hb Constant Spring was confirmed to be associated *in cis* with the neutral SNP α2 +861 G→A.[5]

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 found to be heterozygotes. Double gradient denaturing gradient gel electrophoresis (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-I -5 nt, 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.[1,8,9,10] 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.

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