12-1-2016

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Indian J Hematol Blood Transfus
DOI 10.1007/s12288-015-0617-z

SHORT COMMUNICATION

Detection of α-Thalassemia by Using Multiplex Ligation-Dependent Probe Amplification as an Additional Method for Rare Mutations in Southern Turkey

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Received: 16 March 2015 / Accepted: 4 November 2015

Abstract α-thalassemia is the most common single gene disorder in the Cukurova Region in Turkey. It is therefore routinely screened, including premaritally, in our region. The heterogeneous molecular basis of the disease makes α-thalassemia mutation detection difficult and complex. Besides well established methods, multiplex ligation dependent probe amplification (MLPA) is known as an effective, simple and specific method for the detection and characterization of deletions and duplications. We employed MLPA testing to 30 patients with hematological parameters suggestive of α-thalassemia carrier status but was negative for α-thalassemia with conventional reverse dot blot hybridization (RDB). We found α-globin gene deletions in 3 out of 30 (10 %) patients with MLPA. We propose that MLPA can be used as a second tier test in addition to other techniques such as RDB to identify α-thalassemia carriers in high prevalence regions such as ours, thereby allowing clinicians to provide accurate genetic counselling.

Keywords α-Thalassemia · Multiplex ligation dependent probe amplification · Reverse dot blot hybridization · Southern Turkey

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Introduction

The α-thalassemias are a group of very well-known recessively inherited disorders characterized by hypochromic microcytic anemia [1]. It is the most common hemoglobin disorder, probably the most common human monogenic disorder and is especially frequent in South-East Asia, Mediterranean countries and Africa [2]. It is also known as a global health problem in many aspects, which makes its screening an important public health issue [3].

The clinical phenotype of carriers varies according to the number of genes affected. One dysfunctional α-globin gene causes the individual to be a silent carrier; whereas two α-globin genes deleted/dysfunctional would result in α- thalassemia trait. Hemoglobin H disease (three α-globin genes deleted/dysfunctional) and hydrops fetalis (HbBart's; all α-globin genes deleted); the severe forms of this disease; are the outcomes that most screening programs try to avoid [4].

Genomic deletions involving the α-globin gene cluster comprise the most common molecular cause of α-thalassaemias (80–90 % of cases), while a smaller percentage is caused by point mutations. Misalignment and recombination that alter the number of α-globin genes and represent the primary mechanism for α-thalassemia [5, 6]. To date, a total of 190 and 145 classified mutations were defined on HBA1 and HBA2, respectively, according to Human Gene Mutation Database [7].

A wide range of testing strategies have been used to detect deletions and mutations within the α-globin gene cluster, including Reverse Dot-Blot Hybridisation (RDB), Southern blot (SB), Gap-PCR, multiplex ligation dependent probe amplification (MLPA) and sequence analysis [8–10]. Since the MLPA technique was first described by Schouten et al. [14], many centers have used this method to

Published online: 13 November 2015

identify the α-globin gene cluster deletions [11]. MLPA is a simple technique that allows the detection of any deletions or duplications in the screened regions by rapid quantitative analysis and has been proven to find known and unknown deletions in cases that remain unsolved after performing standard diagnostic techniques [12].

The aim of this study is to evaluate 30 patients suspected to be carriers of α-globin gene mutations, but in whom no mutations were detected by RDB using the MLPA P140-B4 HBA probe mix. Three (10 %) of these patients were found to be α-thalassemia carriers by using this method.

Patients and Methods

Patients

We included 30 patients who were referred to our Genetics Diagnosis Center with microcytic hypochromic anemia, picked up either by the regional premarital screen or via investigation of anemia, between July and October 2014. They all had low mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and normal levels of hemoglobin A2 (HbA2) (1.5–3.5 %), hemoglobin F (HbF) but no detectable α-globin mutation by RDB. Their hematological data is summarized on Table 1. Median age was 9.5 (1–33) and male/female ratio was 1 (15/15). Written informed consents were obtained from all patients. Iron deficiency was excluded as a cause of hypochromic microcytic anemia by evaluating iron and ferritin levels, transferrin iron binding capacity and peripheral blood smear findings. β-thalassemia was excluded for two patients with mildly increased HbA2 levels (P5; HbA2: 4.2 % and P28; HbA2: 4.7 %) by Sanger sequencing of the whole β-globin gene. These two patients were then included in the cohort after excluding other clinical causes, such as HIV therapy or hyperthyroidism.

Hematological Tests

Hemoglobin analysis was carried out by High Performance Liquid Chromatography (HPLC), using the Agilent 1200 Series LC Systems according to manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA). Serum iron levels and unsaturated iron binding capacity (UIBC) were studied with the calorimetric testing method using FerroZine (Sigma Aldrich, USA) on the instrument Cobas 6000 c501 (Roche Diagnostics, Switzerland). Ferritin levels were studied using the electrochemiluminescence/sandwich method on the instrument Cobas 6000 e601 (Roche Diagnostics, Switzerland).

Table 1 Summary of some of the hematological values of our patients

Parameter	Mean value ± SD (range)
RBC ($\times 10^6$/μl)	5.2 ± 0.56 (4.08–6.31)
Hb (g/dl)	10.05 ± 2.02 (6.63–14.6)
MCV (fl)	65.03 ± 7.55 (47.4–77.6)
MCH (pg)	20.45 ± 3.23 (12.9–26.3)
HbA2 (%)	2.34 ± 0.64 (1.50–4.70)
HbF (%)	0.25 (0–3.6)

Reverse Dot Blot Hybridization (RDB)

Total genomic DNA was extracted from 100 μl peripheral blood by High Pure PCR Template Preparation Kit (Roche), according to manufacturer's instructions and stored at −20 °C until genetic analysis was performed. α-globin gene was amplified and biotin-labeled in a single multiplex amplification reaction (Viennelab, StripAssayGmbH, Austria). PCR was performed in a Perkin Elmer 9700 (Singapore) and the protocol consisted of an initial melting step of 2 min at 94 °C; followed by 35 cycles of 15 s at 94 °C, 30 s at 58 °C, and 45 s at 72 °C; and a final elongation step of 3 min at 72 °C. The mutation analysis was performed by Strip Assay technique (ViennaLab, StripAssayGmbH, Austria) which is based on the reverse-hybridization principle automatically.

Multiplex Ligation Dependent Probe Amplification (MLPA)

The assay was performed using the SALSA MLPA kit, P140-B4 HBA (MRC-Holland, the Netherlands) according to the instruction of the manufacturers. A SALSA MLPA Kit, HBA140-B4 (MRC-Holland), was used for α-thalassemia (MRC Holland, 2010). At least three normal control samples were used for each group of patient samples. The MLPA testing was performed as described by White et al. and Schouten et al. [13, 14]. Approximately 200 ng of genomic DNA in a final volume of 5–8 μl was heated for 5 min at 98 °C and cooling it to 25 °C for 1 min. 1.5 ml of the probe mix and 1.5 ml SALSA hybridisation buffer (MRC-Holland) were added to each sample, followed by heat denaturation at 95 °C 1 min sand hybridisation at 60 °C 16–20 h. Ligation was performed by adding 32 μl of Ligase-65 master mix (3 μl of Ligase Buffer A, 3 μl of Ligase Buffer B, 1 μl of Ligase-65 enzyme and 25 μl of dH_2O) to the specimen DNA, which was incubated at 54 °C for 15 min, followed by 95 °C for 5 min. Then the reaction was stopped by cooling to 20 °C. PCR amplification was performed using 10 μl of PCR master mix (0.5 μl of SALSA polymerase, 2 μl of SALSA primer mix, 7.5 μl of dH_2O) and a 40 μl ligated product. PCR conditions were as follows: 35

cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s, followed by a 20 min final extension at 72 and a 15 °C hold. A size standard [0.2 µl (LIZ) GS 500] was added to each sample. PCR products were detected and quantified by capillary electrophoresis in an ABI 3130 genetic analyzer (AppliedBioSystems). The MRC-Coffalyser software (MRC-Holland, The Netherlands) was used as analysis tool for the normalization of MLPA data. The expected results of allele copy numbers of 0, 1, 2, 3, 4 (normal), corresponded to probe ratios of 0, 0.25, 0.5, 0.75 or 1.0 respectively.

Results

RDB showed clearly negative results for α-thalassemia carrier status for all 30 patients. An α-globin gene deletion was detected in 3 (P6, P7 and P16) out of 30 patients (10 %) using MLPA as an additional method. The complete blood count and hemoglobin analysis by HPLC of the patients with deletions are listed in Table 2. The deleted regions of HBA gene for these patients are shown in Fig. 1. In all 3 patients, the probe ratios in the region were around 0.5, which corresponded to 2 allele copy numbers being affected, according to the criteria set by the manufacturer of the MLPA kit.

The MLPA results revealed that the deleted regions expanded from 3.5 kb upstream HBZ to 0.2 kb downstream HBA1 (20 probe) in the two unrelated patients P6 and P7. Whereas the deleted regions expanded from 3.5 kb upstream HBZ to 0.5 kb downstream HBA1(21 probe) in patient 16 (Fig. 2a, b).

Discussion

The molecular testing of α-thalassemia has been challenging due to this complex molecular basis. Previously PCR based methods such as RDB, multiplex PCR, quantitative real-time PCR were used extensively as diagnostic tools [14]. As MLPA is a convenient alternative method for detecting deletions it has recently been a compelling choice for mutation analysis of α-thalassemias [15].

The estimated incidence of α-thalassemia in our region, Cukurova in Southern Turkey is 3 % [16]. Premarital screening for thalassemias is routinely done in Cukurova, as well as other regions in Turkey due to the high prevalence of the disease in the region. Genetic counseling can be difficult in cases where thalassemia trait has been shown clinically and hematologically but cannot be confirmed molecularly. It is crucial to identify the exact molecular change using additional testing to be able to offer prenatal testing to families.

Techniques like Sanger sequencing and denaturing high pressure liquid chromatography that are used the identify point mutations are usually unsuccessful in detecting copy number changes [17]. SB, which is still commonly used to detect α-thalassemia mutations, is a notoriously time consuming technique that falls short to find small deletions. Fallah et al. [11] reported a relative quantitative PCR based

Table 2 The hematological data of the individuals with α-globin gene deletions

Patients	Hb (g/dl)	MCV (fl)	MCH (pg)	RBC (10^6/µl)	HbA2 (%)	HbF (%)
P6	10.3	61	19.6	5.26	2.2	0
P7	10.8	57.6	19.6	5.52	1.7	0
P16	12.1	64.4	20.2	5.99	2.2	0

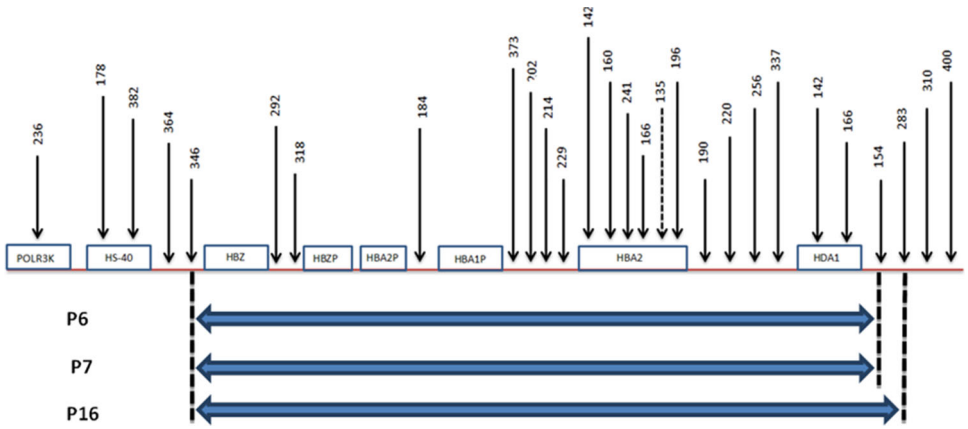

Fig. 1 The schematic representation of 27 probes of MLPA P140-B4 kit and deleted regions of HBA gene in individuals with deletions, designated as *P6*, *P7* and *P16*

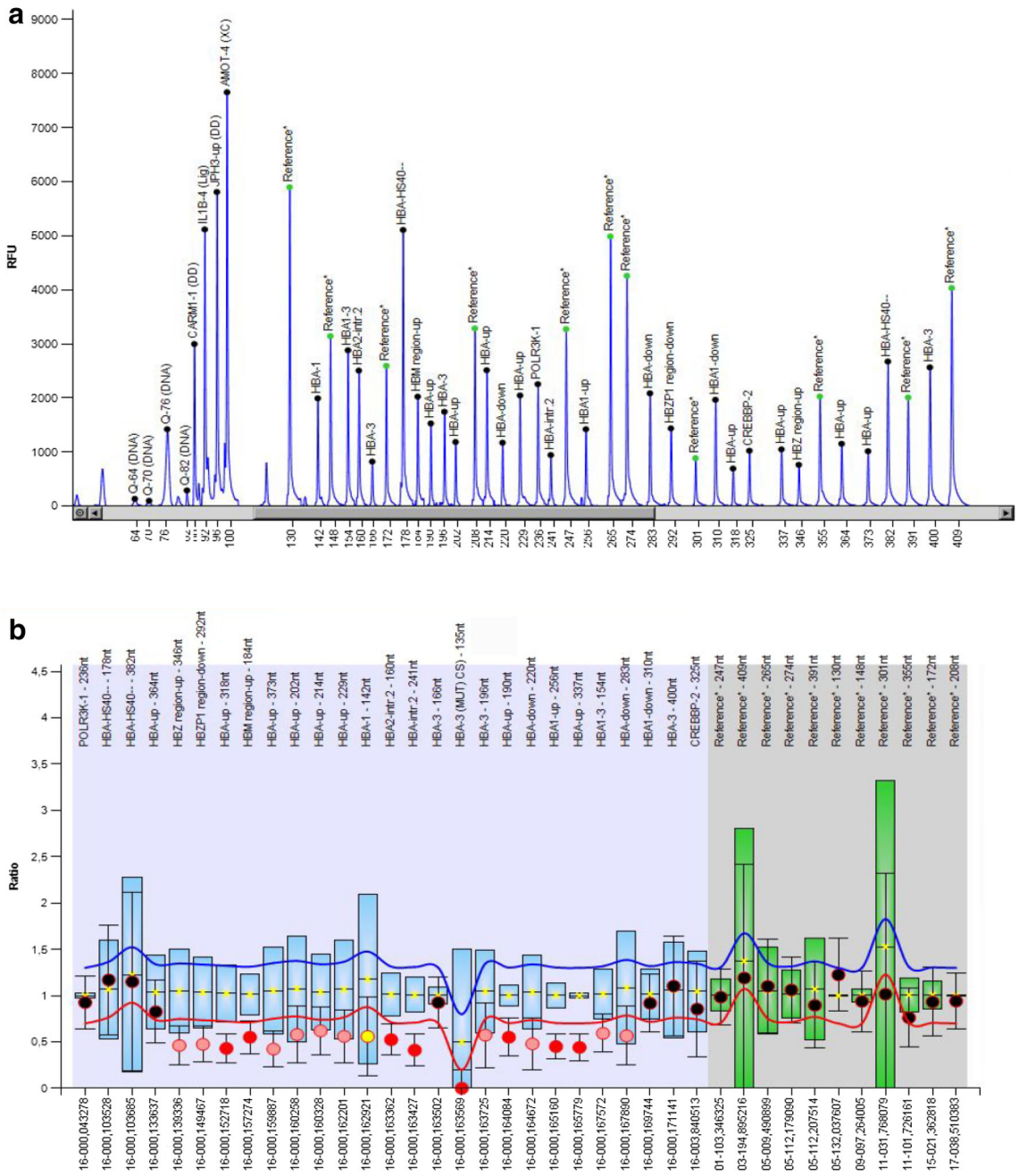

Fig. 2 The MLPA results revealed that the deleted regions expanded from 3.5 kb upstream HBZ to 0.2 kb downstream HBA1 (20 probe) in the two unrelated patients P6 and P7. Whereas the deleted regions expanded from 3.5 kb upstream HBZ to 0.5 kb downstream HBA1 (21 probe) in patient 16 (**a**, **b**)

method as an effective diagnostic tool for identifying unknown α-thalassemia deletions. Sanger sequencing of the critical region is sensitive for detecting mutations in the α-globin gene cluster, but it is relatively expensive, also time consuming and unable to detect large deletions. Therefore while different methods have been trialed for α-thalassemia mutation screening, there are still difficulties in detection, especially of rare or novel deletions.

Even though several studies have been reported comparing MLPA with other methods in α-thalassemia screening, the routine clinical laboratory use of MLPA is still being questioned. One should consider limitations of the technique itself, including the need to purchase additional equipment, commercial kits and supplies, and the occasional requirement to repeat and confirm equivocal results with more conventional methods. However, Kipp

et al. [10] reported a study comparing MLPA and SB Analysis in α-thalassemia and emphasized the importance of MLPA in defining patogenic mutations as a complimentary method to SB, rather than a stand alone technique. Colosimo et al. [8] also identified MLPA as an effective method for screening α-thalassemia mutations. They pointed to the limitations of RDB and suggested that MLPA is an appropriate option to the more time-consuming, labor intensive methods such as SB or Gap PCR [8].

Our study further confirms the functionality of MLPA in the molecular detection of α-thalassemia carriers by revealing mutations in 3 out of 30 individuals (10 %) who were initially deemed negative with RDB. The common 21 mutations screened with our RDB kit do not include the deletions in these patients, which means it would not have been possible to detect a significant portion of carriers in our population with this technique alone. The remaining 27 patients (90 %) could in theory be carriers of cryptic or rare α-thalassemia mutations which would not be detected by RDB or MLPA, but this is less likely. Therefore, while keeping in mind that our sample size is small, we feel that 10 % is a fair representation of a group of patients routinely missed by RDB in our population.

Identifying novel copy number variants in the α-globin gene cluster with the MLPA method and detecting their prevalences in different populations may provide valuable information for commercial probe design by allowing panels to expand. It may even lead to the design of region specific panels and thereby improve the already existing screening techniques in countries with increased prevalence.

The small number of patients in our cohort is one of the limitations of our study and there certainly is a need for studies comparing methods in larger populations in Turkey. Also, since RDB is a standardized and optimized method for our laboratory and controls were unambiguous, we safely assumed that the initial results were not falsely negative. This is expected, since MLPA results showed deletions that could not have been picked up by our RDB kit in the first place. On the other hand, the MLPA results of the three patients could have been confirmed with an additional method. We also cannot comment on the zygosity of the deleted regions for these three patients due to the limitations of the MLPA method. However, for the purposes of this study, we did not feel the need to explore the results further. The value of using additional methods, such as gap PCR and/or Sanger sequencing of the whole α-globin gene as second tier test could also be discussed.

However, using only MLPA as a complementary method for α-globin deletions in our patient group yielded a substantial increase in detection of carriers and hence provided more accurate information to guide genetic counselling in this case series. We suggest that incorporating MLPA as a second tier testing in routine laboratory practice in endemic regions, such as Southern Turkey, in individuals with hematological evidence of α-thalassemia trait may increase detection rates and create more options for prenatal diagnosis. For the rarer cases where combined RDB and MLPA testing is still not able to detect carriers with certainty, additional methods such as Sanger sequencing of α-globin gene should be performed.

Incorporating this method will hopefully aid our efforts to ameliorate the screening and management of α-thalassemia as a challenging public health issue in our region.

Acknowledgments We are thankful to the patients for their agreement to participate in this report.

Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

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