Setup of a Protocol of Molecular Diagnosis of β -Thalassemia Mutations in Tunisia using Denaturing High-Performance Liquid Chromatography (DHPLC)

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Backgrounds: β -Thalassemia is one of the most prevalent worldwide autosomal recessive disorders. It presents a great molecular heterogeneity resulting from more than 200 causative mutations in the β -globin gene. In Tunisia, β -thalassemia represents the most prevalent monogenic hemoglobin disorder with 2.21% of carriers. Efficient and reliable mutation-screening methods are essential in order to establish appropriate prevention programs for at risk couples. The aim of the present study is to develop an efficient method based on the denaturing high-performance liquid chromatography (DHPLC) in which the whole β -globin gene (HBB) is screened for mutations covering about 90% of the spectrum. **Methods:** We have performed the validation of

a DHPLC assay for direct genotyping of 11 known β -thalassemia mutations in the Tunisian population. **Results:** DHPLC assay was established based on the analysis of 62 archival β -thalassemia samples previously genotyped then validated with full concordance on 50 tests with blind randomized samples previously genotyped with DNA sequencing and with 96% of consistency on 40 samples as a prospective study. **Conclusion:** Compared to other genotyping techniques, the DHPLC method can meet the requirements of direct genotyping of known β -thalassemia mutations in Tunisia and to be applied as a powerful tool for the genetic screening of prenatal and postnatal individuals. J. Clin. Lab. Anal. 30:392–398, 2016.

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INTRODUCTION

β -Thalassemia, one of the most prevalent worldwide autosomal recessive disorders, is characterized by a reduced production of functional β -globin chain, leading to anemia. Despite the great heterogeneity of β -thalassemia molecular spectrum where more than 200 causative mutations are reported worldwide, it has been noted that in each geographical area—with elevated prevalence of β -thalassemia—a limited number of five to six alleles was responsible of more than 80% of the spectrum (1).

The geographical position of Tunisia between the sub-Saharan African and Mediterranean countries makes it well concerned by β -thalassemia with a frequency estimated at 2.21% (2). This high prevalence justifies the establishment of a molecular investigation protocol that would be of a great interest for genetic

counseling or prenatal diagnosis offered to at increased risk families.

Various methods for screening of the known causative mutations of β -thalassemia have been reported, including amplification refractory mutation system (ARMS) (3), allele-specific oligonucleotide (ASO) blot (4), reverse dot blot (RDB) (5), microarrays (6), fully denaturing

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HPLC with primer extension (7), ligase chain reaction (LCR) (8), pyrophosphorolysis-activated polymerization (PAP) technology (9), microtiter well-based chemiluminometric genotyping assay (10), and high-throughput systems based on matrix-assisted laser desorption/ionization (MALDI)-time-of-flight (TOF) mass spectrometry (11). However, none appears ideal for rapid screening of a large number of samples. The most important issues are detection of as many mutations as possible, cost effectiveness, rapidity and accuracy in obtaining the diagnosis, and eventually the ability to handle large numbers of samples by automated methods. Methodology should be also simple enough to be used in clinical laboratories to detect the most prevalent β -thalassemia mutations and to be applied in other populations groups.

To address some of these issues, we have attempted in the present study to develop an efficient method based on the denaturing high-performance liquid chromatography (DHPLC) in which the whole β -globin gene (HBB) is screened for 11 β -thalassemia mutations. The choice of these mutations was based on their prevalence in the Tunisian population, knowing that they represent about 92.1% of the spectrum (12).

MATERIAL AND METHODS

Subjects

DHPLC assay conditions were developed using 62 archival samples of DNA previously genotyped by RDB and automated sequence analysis (12) as heterozygous β -thalassemia ($n = 47$) or β -thalassemia major ($n = 15$). Five DNA samples from healthy control subjects were used as wild type for β -thalassemia genotypes.

The mutations tested in this study were $-5'$ to $3'$ on the HBB gene: -30 (T-A), -87 (C-G), codon 6 (-A), codon 30 (G-C), codon 39 (C-T), codon 44 (-A), IVS-1-1 (G-A), IVS-1-5 (G-C), IVS1-6 (T-G), IVS1-110 (G-A), and IVS2-745 (C-G). A validation of set-up conditions was performed on 50 tests with blind randomized samples that were homozygous or heterozygous of the studied β -thalassemia mutations as previously determined by direct sequencing analysis. The DNA sequencing results were unknown until the genotypes obtained from each of the given DHPLC profile were scored.

A prospective study conducted in a cohort of 40 unrelated β -thalassemia patients was performed to evaluate the validity of DHPLC.

EXPERIMENT DESIGN

DNA Extraction

Human genomic DNA was isolated from leukocytes in peripheral blood using standard DNA extraction method.

PCR Conditions

The process developed for the identification of β -thalassemia mutations was based on the amplification of three DNA fragments followed by DHPLC analysis. Three sets of primers were designed to amplify the three fragments that encompass regions of known mutations occurring in β -globin gene in the Tunisian population. Primer sequences are shown in Table 1.

The polymerase chain reaction (PCR) reactions were performed in a total volume of 50 μ l containing 200 ng of genomic DNA, 0.8 μ M of each primer, 0.1 mM of each dNTPs, 1 U of polymerase enzyme, and 10 μ l of 5 $\times$ buffer using the Applied Biosystems Gene Amp[®] PCR System 9700 thermal cycler under the following conditions: an initial denaturation step at 94°C for 5 min, 30 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, and followed by a final extension at 72°C for 5 min.

DHPLC Analysis

Prior to DHPLC analysis, heteroduplexes were obtained by denaturing the PCR products at 94°C for 10 min and cooling at 56°C for 60 min. DHPLC analysis, a reverse-phase ion-pair high-performance liquid chromatography allowing identification of heteroduplex molecules, was performed on the Transgenomic Wave Nucleic Acid Fragment Analysis System (Transgenomic, Inc., San Jose, CA). Eight microliter of PCR product was injected for each run. Elution from the cartridge was performed using a linear acetonitrile gradient in an 8.8 min sample run in 0.1 mM triethylamine acetate buffer (TEAA; Transgenomic), pH 7 at a constant flow rate of 0.9 ml/min. The gradient was formed by mixing buffer A (0.1 mM TEAA) and buffer B (0.1 mM TEAA, 25% acetonitrile). The temperature of the oven for optimal heteroduplex separation with partial DNA denaturation was deduced from the melting profile of the DNA sequence obtained with Wavemaker[®] 3.4.4 software (Transgenomic). The eluted products were detected by UV analysis at 260 nm. Heterozygous profiles were identified by visual inspection of the chromatograms on the basis of the appearance of additional earlier eluting peaks, whereas homozygous profiles showed only one peak.

Data Analysis

Two researchers interpreted the results independently. Five wild-type samples were always used as negative controls to ensure that a normal homoduplex profile was reproducibly obtained taking into consideration retention time and peak profile. Samples with extra peaks (one, two, or three more), or with a difference in peak appearance, were scored as positive.

TABLE 1. Sequences of Oligonucleotide Primers and Analytical Conditions Used in DHPLC Assay

Fragments	β -Thalassemia mutations	Primer sequence (5'→3')	Size (bp)	DHPLC conditions	
				Oven temperatures (°C)	Initial concentration of buffer B (%)
1	–30 (T→A), –87 (C→G), Cd6 (–A)	5'GTCCAACCTCCTAAGCCATGG3'	291	60.8, 61.1	54.4
2	Cd30 (G→C), IVS1-1 (G→A), IVS1-6 (T→C), IVS1-110 (G→A), Cd 39 (C→T) and Cd 44 (–C)	5'CAACTTCATCCACGTTTACC3' 5'GTACGGCTGTCATCACTTAGACCTCA3'	491	62.5, 63, 63.5	50
3	IVSII-745 (C→G)	5'ATCAGGATGTGGACAGATCCC 3' 5'GTGTACACATATTGACCAAA 5'GCACACAGACCAGCACGTTG 3'	422	55.5, 56	57.4

Each sample showing positive profile was reanalyzed after being mixed with an equal amount of heterozygous type of the same mutation (denatured and reannealed). Of the same, subjects presenting one peak were mixed with wild-type DNA.

RESULTS

In order to set up elution conditions and chromatography profiles of 11 known β -thalassemia mutations, we ran amplicons obtained from DNAs with heterozygous or homozygous β -thalassemia mutations previously identified by RDB or sequencing analysis. The mutations tested were codon 39 (C→T), IVS1-110(G→A), IVS-1-1 (G→A), IVS-1-5 (G→C), IVS1-6 (T→C), codon 44 (–A), codon 30 (G→C), IVS2-745 (C→G), codon 6 (–A), –30 (T→A), and –87(C→G). The gradient and temperature conditions are shown in Table 1.

In the first amplicon, we noted that under the same denaturing temperature (61.1°C) and gradient (54.5% B), three characteristic chromatography profiles were obtained for the mutations –30, –87, and codon 6 with different retention times 4.3, 5.5, and 4.5, min, respectively (Fig. 1A).

For the second amplicon, codon 30, codon 39, codon 44, IVS1-1, and IVS1-6 mutations revealed different chromatograms under the same gradient of acetonitrile (50% B). If we consider at the same time the denaturation temperatures (63.5°C, 63°C, 63.5°C, 62.5°C, and 61.8°C, respectively), the retention times (3.5, 4, 3.5, 3.5, and 4.3 min, respectively), and the aspects of chromatograms,

we find that each of these profiles is characteristic of the tested mutation. However, we noted identical profiles and retention times for the heterozygous IVS1-5 and IVS1-110 mutations at the same denaturing conditions (50% B and 63.5°C), making their identification likely to be confused. On the other hand, we noted that the IVS1-5 mutation had another positive profile at the 64°C making thus its identification possible at this temperature (Fig. 1B).

In the third amplicon, we observed a slight denaturation for the IVSII-745 mutation at 55.5°C, but when increasing the temperature to 56°C, we noted a better separation of the heteroduplexes and homoduplexes with the same gradient of acetonitrile (57.4% B) and retention time (5.8 min; Fig. 1C).

Under each DHPLC condition retained for the 11 most common mutations among the Tunisian population, wild-type controls showed a unique peak corresponding to the normal allele. Homozygous mutations specimens—when tested—revealed patterns not distinguishable from wild-type controls in all tested conditions. However, the same elution patterns—as the heterozygous corresponding mutation—were found when a heteroduplex was performed with the PCR product of a wild-type DNA.

Hence, from the amplification of three DNA fragments of size between 300 and 500 bp (ideal sizes for DHPLC analysis), we developed a rapid method for identification of β -thalassemia mutations based on the nature of the chromatographic profiles of the homoduplexes and heteroduplexes generated by DHPLC aspect, retention time, denaturation temperature, and denaturing gradient.

Fig. 1. Representative DHPLC profile patterns of fragments 1, 2, and 3 for genotyping of β -globin gene mutations: -87, -30, and Cd6 (A), Cd30, IVSI-1, IVSI-5, IVSI-6, IVSI-110, Cd39, and Cd44 (B), IVSII-745 (C). In each fragment the normal DHPLC profile of wild type (WT) is also presented.

TABLE 2. A List of DHPLC Results Observed From DNA Samples Tested in the Study

First amplicon				Second amplicon				Third amplicon			
Genotype	Setup tests	Blind tests	Prospective tests	Genotype	Setup tests	Blind tests	Prospective tests	Genotype	Setup tests	Blind tests	Prospective tests
–87°/N	2	1	–	Cd30/N	4	3	2	IVSII-745°/N	4	1	2
–87/–87	–	1	–	Cd30/Cd30	5	–	–	IVSII-745°/IVSII-745	1	–	–
–30°/N	2	2	1	IVSI-1/N	3	4	2				
–30/–30	1	1	–	IVSI-1/IVSI-1	–	2	1				
Cd6/N	3	2	1	IVSI-5/N	3	4	–				
Cd6/Cd6	–	–	–	IVSI-5/IVSI-5	1	–	1				
				IVSI-6/N	1	3	2				
				IVSI-6/IVSI-6	–	–	–				
				IVSI-110/N	4	8	7				
				IVSI-110/IVSI-110	1	1	2				
				Cd39/N	17	9	10				
				Cd39/Cd39	8	4	4				
				Cd44/N	4	3	5				
Total	8	7	2		51	41	36		5	1	2

To further test the DHPLC genotyping assay, we performed 50 blind tests randomly chosen from samples that were homozygous or heterozygous for the 11 studied mutations (Table 2). The genotypes were 100% in concordance with the results of DNA sequencing.

We also validated the accuracy of this DHPLC method against 32 DNA samples from β -thalassemia minor individuals and eight β -thalassemia major patients. DHPLC assays included heteroduplex of the tested DNA itself or after mixing with the wild-type control. The order in screening for β -thalassemia mutations was based on previously reported genotype frequencies (Table 3) with this workflow: cd 39 (C→T), IVSI-110 (G→A), IVSI-1 (G→A), cd 44(-C), cd 30 (G→C), IVSII-745 (C→G), cd 6 (–A), –87 (C→T), IVSI-5 (G→C), –30 (T→A), and IVSI-6 (T→C). The DHPLC analysis allowed the identification of the β -thalassemia genotype in 46 chromosomes among the 48 studied ones. The allelic frequencies among the 40 samples compared to those described in previous studies are reported in Table 3.

DISCUSSION

β -Thalassemia is one of the most common genetic diseases in the world. It is an autosomal recessive inherited hemoglobinopathy resulting from point mutations, small insertions, or deletions in the β -globin gene (HBB) (13–15). The clinical phenotype and disease severity are variable but often predictable from the molecular defect, making the identification of β -thalassemia mutations an essential step in the early management of patients. It is also a prerequisite for β -thalassemia carriers screening (16) in prevention programs and prenatal diagnosis offered to at risk couples (2). In Tunisia, β -thalassemia mutations

spectrum revealed a great molecular heterogeneity with 28 different alleles reported up to now (12, 13, 17, 18).

A wide variety of methods for genotyping the HBB gene have been developed using different techniques, including those studying known mutations, such as allele-specific oligonucleotide hybridization (ASO), amplification refractory mutations system (ARMS), allele-specific PCR, RDB, restriction fragment length polymorphism (RFLP), and single-base extension (SBE), and others screening unknown mutations, such as single-strand conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE).

During the last few years, ARMS, RDB, and RFLP were the principle methods used for β -thalassemia alleles identification in previous Tunisian studies (12, 14). These methods based on different principles allow the identification of previously reported alleles. Thus, ARMS, which is a PCR-based method using allele-specific primers, consists in performing for each tested mutation two independent PCR reactions of both the normal and the mutant alleles with two couples of primers. Despite the reproducibility of this method, we may note false-positive results caused by as simple modifications in operating conditions as changing the controls or the thermocycle. As for the RDB reaction, which is also an allele specific hybridization-based method, we note that its main advantage is that the same amplified DNA can be tested for many mutations at the same time. However, the difficult operating conditions in the setup of this method may reduce its efficiency particularly in situations requiring a rapid and accurate diagnosis, such as the prenatal one. On another hand, based on the principle of recognition of restriction sites, the PCR-RFLP method allows only the determination of a limited number of molecular defects (19).

TABLE 3. Analytical Orders and Frequencies of the Tested β -Thalassemia Mutations in the Prospective Study and in a Previous Tunisian Study

Analytical orders	Mutant type	Number of chromosomes (<i>n</i>)	Frequency (%)	Frequency according to Fattoum (2) (%)
1	cd 39 (C→T)	18	37.5	49
2	IVSI-110 (G→A)	11	18.75	21
3	IVSI-1 (G→A)	4	8.33	4.5
4	cd 44 (-C)	5	—	3.8
5	cd 30 (G→C)	2	4.16	3.2
6	IVSII-745 (C→G)	2	4.16	2.6
7	cd 6 (-A)	1	2.08	2.6
8	-87 (C→T)	—	—	1.7
9	IVSI-5 (G→C)	2	4.16	1.5
10	-30 (T→A)	1	2.06	0.8
11	IVSI-6 (T→C)	2	4.16	0.6

Concerning other methods allowing the screening for unknown mutations, we note that the SSCP is a simple, fast, and low-costing method. Its efficiency is influenced by various factors, such as the size and the nucleotide composition of the amplified fragment, the percentage of polyacrylamide, and also the temperature and time of electrophoresis. It is notable that to attend high sensitivity with this method, the size of the amplified DNA may not exceed 300 bp (20). Compared to the DGGE, the SSCP may present some difficulties of interpretation and a lower percentage of detection, which limit its use. In fact, DGGE has been widely used in the investigation of molecular lesions associated with β -thalassemia (18). The sensitivity of detection of mutations is more than 95% and the size of analyzed fragments may reach 500 bp. Nevertheless, this method is based on addition of clamped sequences in 5' extremity of the amplified fragment, which significantly increases the cost of primers.

The request for rapid and reliable β -thalassemia mutations identification method is constantly increasing for the definition of clinical samples and orientating targeted therapies or counseling (21).

The integration of results from DHPLC patterns in the prospective study population enabled us to identify the causative mutations in 96% of cases. This percentage is better than those reported in previous Tunisian's studies using more onerous techniques of molecular biology, such as SSCP, DGGE, ARMS, and sequencing analysis (13, 22), where the percentage of mutations identified was estimated at 76.19% and 67.77%, respectively. Furthermore, the different allelic frequencies we noted are in perfect agreement with results reported in previous studies among Tunisians (12, 13, 22).

The ability of DHPLC method to detect known mutations has been successfully applied for the study of several disease-causing alleles and the results showed sensitivity and specificity approaching 90–100%. Its efficiency in the

study of β -globin gene has been proved in former studies among other ethnic populations, such as Chinese and Thai (21, 23). These data prove that this method can be implemented in different at risk of hemoglobinopathies populations, such as the Tunisian one. It does not require any particular pre- or post-PCR manipulation reagents. We noted that the DHPLC offered an excellent accuracy and reproducibility given that identical DNA samples revealed similar patterns each time they were treated in the same denaturing conditions. The reproducibility of the elution chromatograms has been validated by several other studies (21, 24). Moreover, compared with other current methods, such as sequencing analysis, the cost per test, including reagents and consumables for the DHPLC assay, is less expensive making this technique an attractive option for rapid genotyping.

All of these characteristics make of DHPLC among the most flexible methods for both postnatal and prenatal molecular diagnosis of β -thalassemia (25). In extreme cases, when the elution profile is not easily ascribable to known common mutations, it is mandatory to perform direct sequencing to discriminate between previously unreported mutations, neutral polymorphisms, and new mutations.

In Tunisia and other areas with high prevalence of β -thalassemia, the adoption of the DHPLC-based format should be an excellent new method to be applied for genetic screening and prenatal diagnosis. In some previous studies, authors have even suggested for high-throughput applications for DHPLC patterns, an automatic genotype assignment using a software program (21).

However, this method requires a setup of different operating conditions. Elution profile differences are due to alterations in oven temperatures, variations in the amount of PCR product, changes in the conditions of the DHPLC buffer, and the quality of instrument maintenance. Therefore, slight changes in the experimental conditions

can produce false-negative and false-positive test results. Optimization requires also at least three control DNA (homozygous, heterozygous, and wild type) for the tested mutation.

Ethics

The work performed in our study is in accordance with the Ethical Guidelines of World Medical Association Declaration of Helsinki and has been approved by the Ethical Committee of the Children's Hospital of Tunis.

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CONFLICT OF INTEREST

The authors give full disclosure of any conflict of interest.

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