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MOLECULAR DIAGNOSTICS OF β -THALASSEMIA

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ABSTRACT

A high-quality hemoglobinopathy diagnosis is based on the results of a number of tests including assays for molecular identification of causative mutations. We describe the current diagnostic strategy for the identification of β -thalassemias and hemoglobin (Hb) variants at the International Reference Laboratory for Haemoglobinopathies, Research Centre for Genetic Engineering and Biotechnology (RCGEB) “Georgi D. Efremov,” Skopje, Republic of Macedonia. Our overall approach and most of the methods we use for detection of mutations are designed for the specific target population. We discuss new technical improvements that have allowed us to substantially reduce the average time necessary for reaching a conclusive diagnosis.

Keywords: Hb Lepore; Hemoglobinopathy; Molecular diagnostics; Thalassemia.

INTRODUCTION

Hemoglobinopathies are caused by genetic defects affecting the globin genes encoding for the α and β chains of the hemoglobin (Hb) molecule. In the Mediterranean region in particular, there is a high incidence of mutations disturbing the function of the *HBB* gene [1]. Some of the mutations reduce or eliminate the expression of the *HBB* gene leading to net Hb deficiency and β -thalassemia (β -thal) [2]. Other mutations give rise to abnormal Hb variants such as Hb S [$\beta 6(A3)Glu \rightarrow Val$, *GAG > GTG*] and Hb Lepore-Boston-Washington (Hb LBW; $\delta 87-\beta IVS-II-8$) [2-4]. The severity of the clinical symptoms depends on the molecular consequences of the genetic abnormality or combination thereof and is modulated by other genetic and environmental factors [2,5,6].

The best practice in hemoglobinopathy diagnostics involves molecular identification of the causative mutations. Molecular diagnostics of β -thal has been a major focal point of the activities at the International Reference Laboratory for Haemoglobinopathies, Research Centre for Genetic Engineering and Biotechnology (RCGEB) “Georgi D. Efremov”, Skopje, Republic of Macedonia at Skopje, Republic of Macedonia. Thousands of cases have been tested for the presence of mutations throughout the years. This study has helped determine the frequencies of the most common

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mutations in Macedonia and several neighboring countries [7-10]. Until recently, the primary method for the detection of β -thal mutations in our laboratory was allele-specific oligonucleotide hybridization, whereby consecutive rounds of hybridization with several mutation-specific probes were performed. We were looking to reduce the time necessary for reaching a definitive diagnosis by introducing a semi-automated technique allowing simultaneous detection of the most commonly occurring β -thal mutations. Unfortunately, published techniques, such as primer extension and melting curve analysis were suboptimal in terms of precision and multiplexing [11-13]. We therefore set out to develop a new assay for the identification of common Mediterranean mutations that occur at high frequencies in the Republic of Macedonia, namely HBB:c.93-21G>A, HBB:c.92+1G>A, HBB:c.92+6T>C, HBB:c.118C>T, HBB:c.316-106C>G, HBB:c.17_18 del CT, HBB:c.20delA, HBB:c.25_26delAA and HBB:c.20 A>T [14,15]. Our protocol utilizes single-nucleotide primer extension to interrogate the whole panel of mutations in a single, internally controlled reaction. We also designed a complementary duplex polymerase chain reaction (PCR) assay for detection of the Hb LBW deletion NG_000007.3:g.63632_71046 del, the most common Hb variant in our geographic area [9]. Both assays have been thoroughly validated and evaluated as described elsewhere (Atanasovska *et al.*, in preparation). Here we report the overall diagnostic strategy for the identification of β -thal mutations and Hb variants that includes the new assays.

MATERIALS AND METHODS

The human subjects that participated in this study were referred to the RCGEB for laboratory investigations for suspected haemoglobinopathies. Informed consent was obtained in accordance with the Declaration of Helsinki. Peripheral blood samples were obtained by standard venipuncture. Standard blood test results were obtained for each patient. Levels of Hb A, Hb A₂, Hb F and Hb variants were measured by cation exchange high performance liquid chromatography (HPLC) [16]; red cell osmotic fragility was assessed by the osmotic fragility test as described before [17]. Genomic DNA was isolated following a standard phenol extrac-

tion/ethanol precipitation protocol. DNA was dissolved in TE buffer, pH 8.0 and stored at 4°C. The multiplex single-nucleotide primer extension assay for detection of Mediterranean β -thal mutations and the duplex PCR assay for detection of the Hb LBW deletion were performed as described elsewhere (Atanasovska *et al.*, in preparation). A subset of samples were analyzed by sequencing of the PCR-amplified *HBB* gene using the ABI PRISM™ Big Dye Terminator v.1.1 Kit (Life Technologies, Carlsbad, CA, USA). The sequences of the primers used for PCR amplification and sequencing are available upon request.

RESULTS AND DISCUSSION

The vast majority of subjects referred to the laboratory were heterozygous carriers of hemoglobinopathy mutations and non carriers referred by their medical specialist or general practitioner to exclude a hemoglobinopathy. Our diagnostic algorithm is presented in Figure 1. When a case is referred to the laboratory for hemoglobinopathy examination, we initially review the patient data including standard blood test results and family history and run a second tier of hematology tests, namely HPLC quantification of normal and abnormal Hb variants and assessment of red cell osmotic fragility. If a Hb variant, *e.g.*, the relatively common Hb LBW, is present in a sample it is identified on the HPLC profile at this stage by virtue of its specific retention time, proportion of total Hb and peak characteristics. Samples identified as Hb LBW heterozygotes or homozygotes by HPLC are tested by the Lepore PCR assay to confirm the presence of the Hb LBW chromosome. This approach warrants unequivocal identification of Hb LBW cases at an early stage of the procedure. These samples are then excluded from further analysis unless a severe clinical picture suggests compound heterozygosity (see below).

Thalassemia is diagnosed based on red blood cell indices combined with the results of the osmotic fragility test and the HPLC analysis. The main diagnostic parameters pointing to probable β -thal trait are: elevated Hb A₂, low total Hb level, low mean corpuscular volume (MCV), low mean corpuscular Hb (MCH), elevated Hb F, decreased osmotic fragility. β -Thalassemia symptoms can vary between carriers, *e.g.*, an individual could feature most if

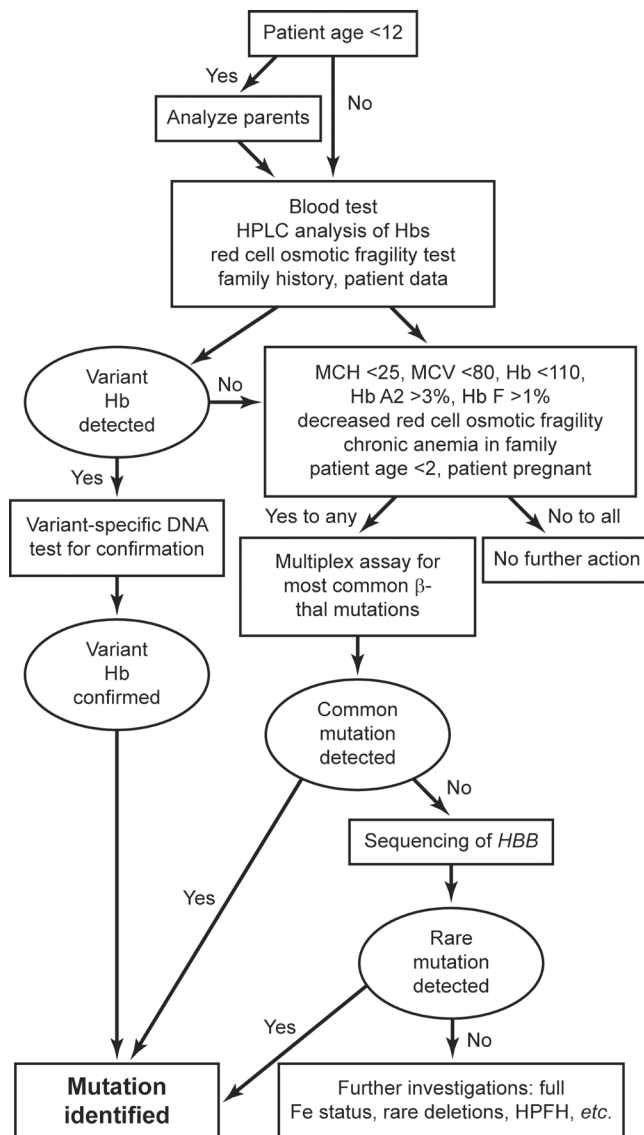


Figure 1. Flow chart of the diagnostic algorithm for identification of patients carrying *HBB* mutations.

not all of these indicators to almost none or borderline values. Using several independent parameters minimizes the risk of missing β -thal carriers in the initial screen. The clinical picture for homozygotes and compound heterozygotes is largely clearer and there is a much lower risk of misdiagnosing these cases.

Based on the results of these analyses, we assign cases for molecular detection of β -thal trait. These samples are first tested for the presence of the eight most common β -thal mutations by the multiplex single-nucleotide primer extension assay. In the past, our protocol for molecular characterization of the *HBB* gene was prohibitively time-consuming,

forcing us to apply relatively stringent inclusion criteria. The introduction of the new multiplex assay allows us to test the majority of the subjects referred to the laboratory. In particular, children are tested for the most common mutations by default even if their blood test results are compatible with a normal genotype. Parents are also invited to provide blood samples so that the diagnosis is cross-checked independently. It has to be pointed out that the presence of high levels of Hb F in children younger than 1 year of age can mask the manifestation of β -thal. It is therefore important to apply definitive DNA tests to eliminate false negative results. Collectively, the most common β -thal mutations detected by the multiplex assay account for approximately 90.0% of all hemoglobinopathy cases in the Republic of Macedonia [7,8]. In case the multiplex assay yields a normal genotype while the hematology data points to the presence of a β -thal mutation, the sample is assigned for sequencing of the *HBB* gene in order to reveal genetic variations not tested in the multiplex assay.

Since we incorporated the multiplex assay and the Lepore PCR assay into the routine hemoglobinopathy work-up in our laboratory, we have processed a total of 186 patient samples. For 83 cases, the data from the various hematological tests were concordant and compatible with a normal genotype and further testing was not necessary. Nevertheless, these samples were assayed by the multiplex assay partly to corroborate the absence of common mutations, partly to assess the assay reproducibility. Hemoglobinopathies were thus excluded in these cases. For the remaining 103 cases, there were indications for genetic abnormalities affecting the *HBB* gene. Three of these patients were confirmed to be Hb LBW heterozygotes (Table 1) and were not tested further. We applied the multiplex thalassemia assay to the remaining 100 samples and identified 95 β -thal heterozygotes, each carrying one of the β -thal mutations included in the assay (Table 1). The multiplex assay failed to identify any mutations in five samples. These were subjected to direct sequencing of the *HBB* gene and were found to carry other β -thal mutations (Table 1) in unison with the hematology data. Thus, a conclusive diagnosis was reached for every case. Importantly, the newly developed procedures have significantly reduced the time and cost necessary to complete the analyses.

Table 1. Mutations detected by the multiplex single-nucleotide extension assay, the Lepore polymerase chain reaction assay and direct sequencing of the *HBB* gene.

HGVS Nomenclature ^a	Mutation ^b	Type	Assay	Chromosomes Detected
HBB:c.93-21G>A	IVS-I-110 (G>A)	β^+	Multiplex	37
HBB:c.92+1G>A	IVS-I-1 (G>A)	β^0	Multiplex	11
HBB:c.92+6T>C	IVS-I-6 (T>C)	β^+	Multiplex	15
HBB:c.118C>T	codon 39 (C>T)	β^0	Multiplex	16
HBB:c.316-106C>G	IVS-II-745 (C>G)	β^+	Multiplex	6
HBB:c.17_18delCT	codon 5 (-CT); CCT(Pro)>C--	β^0	Multiplex	3
HBB:c.20delA	codon 6 (-A); GAG(Lys)>G-G	β^0	Multiplex	5
HBB:c.25-26delAA	codon 8 (-AA); AAG(Lys)>--A	β^0	Multiplex	2
NG_000007.3:g.63632_71046del	Hb LBW	Hb+ β -thal	Lepore PCR	3
HBB:c.-80T>A	-30 (T>A)	β^+	Sequencing	2
HBB:c.316-3C>A	IVS-II-848 (C>A)	β^+	Sequencing	2
HBB:c.250delG	codons 82/83 (-G); AAG GGC(Lys Gly)>AAG -GC	β^0	Sequencing	1

^a Patrinos *et al.* [15].

^b Huisman *et al.* [14].

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