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# Invasive and Noninvasive Approaches in Prenatal Diagnosis of Thalassemias

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Abdullah Tuli and Ebru Dünder Yenilmez

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## Abstract

Thalassemia is a significant health problem worldwide. There are two main classifications,  $\alpha$ - and  $\beta$ -thalassemias, which are usually caused by the defective synthesis of the  $\alpha$ -globin, and which are commonly caused by different mutations of the  $\beta$ -globin chain. Different hemoglobin mutations have been identified to date. Thalassemias can result in profound anemia from early life and, if not treated with regular blood transfusions, can lead to death in the first year. Prenatal diagnosis of thalassemia is the essential part of preventive medicine and is currently dependent on the use of invasive diagnostic tests within the first 2 months of pregnancy. These diagnostic techniques carry a small but significant risk of fetal loss up to 1%. Molecular diagnostic methods have been developed for genotyping thalassemias based on PCR techniques and high-throughput technologies. Noninvasive tests using cell-free DNA (cfDNA) from a maternal blood sample is also an alternative method, thus eliminating the risk of miscarriage. This chapter summarizes the current invasive approaches and the noninvasive methods using cell-free fetal DNA as new molecular diagnostic methods for genotypic diagnosis of thalassemia in clinical practice. Prevention strategies that encompass carrier screening, genetic counseling, and prenatal diagnosis are discussed.

**Keywords:** thalassemia, prenatal diagnosis, noninvasive test, cell-free DNA, molecular method

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## 1. Introduction

Hemoglobin (Hb) is the oxygen carrier molecule in red blood cells. Each adult Hb molecule consists of four subunits: two  $\alpha$ -globin and two  $\beta$ - (or  $\beta$ -like) globin chains. The  $\alpha$ -globin gene

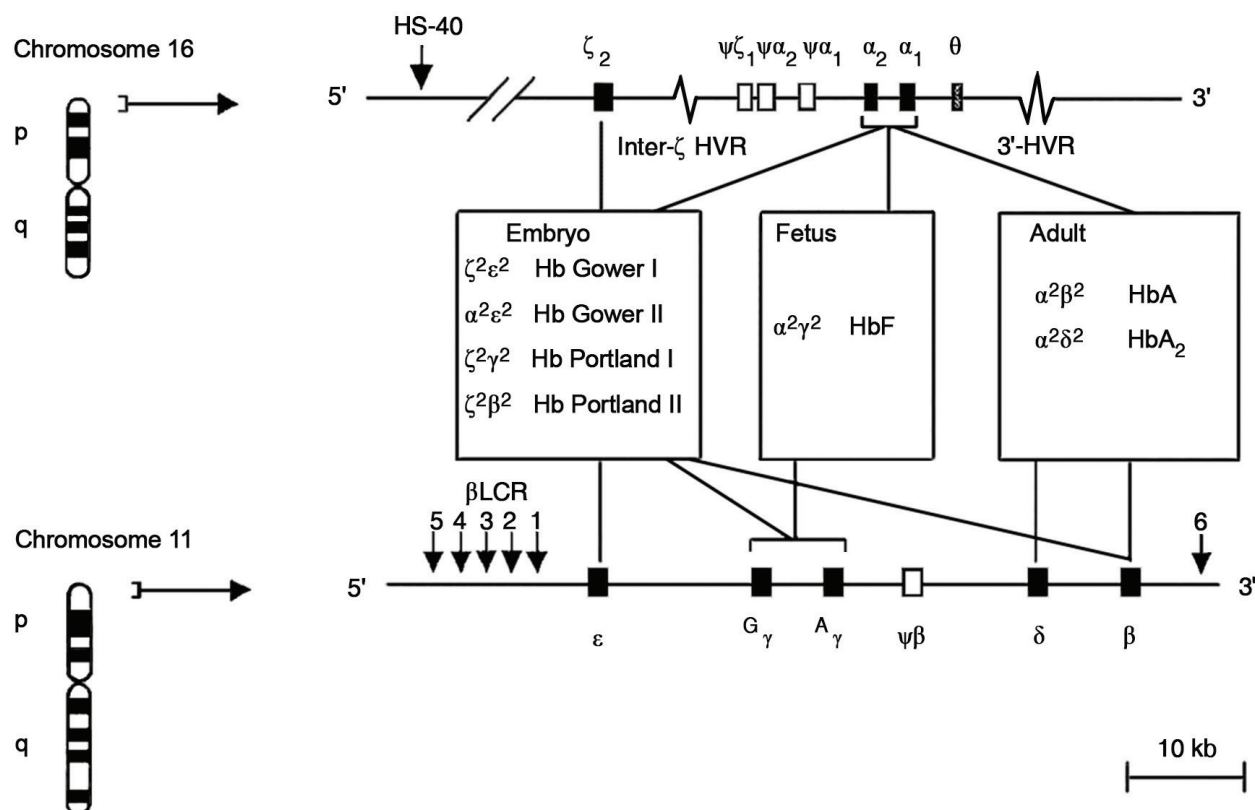


Figure 1. Alpha beta globin gene cluster [4].

cluster maps near the telomere of the short arm of chromosome 16. The human  $\beta$ -globin spans a region of 70 kb on the short arm of chromosome 11 and contains 5 functional genes. Hb A is the predominant form of Hb molecule in an adult human [1–4]. The  $\alpha$ -globin gene cluster undergoes one developmental “switch” but the  $\beta$ -gene cluster undergoes two “switches”. Transcription of the  $\epsilon$  gene in the embryonic gene stage switches after the sixth week of gestation to the transcription of the two  $\gamma$  genes in the fetal liver and around the prenatal period, to the  $\delta$  (minor adult) and  $\beta$  (major adult) genes (**Figure 1**). At 6 months after birth, hemoglobin F (HbF) constitutes less than 5% of the total hemoglobin and continues to fall, reaching the adult level of <1% at 2 years of age [4].

## 2. Hemoglobinopathies

Inherited hemoglobinopathies are large groups of autosomal recessive disorders [4]. There are more than 700 defects in globin genes that are found to be responsible for hemoglobinopathies [5]. The defective (+) or absent (0) production of one of the globin chains of the Hb tetramer causes autosomal recessive inherited disorders. The type of globin chain involved distinguishes  $\alpha$ -,  $\beta$ -, and  $\delta$ -thalassaemias [6]. The meaning of thalassemia comes from the Greek words *thalassa*; sea and *aemia*; anemia. It was observed that thalassemia is prevalent in areas in which malaria was seen or was endemic [7, 8].

The resulting imbalance in the ratio of  $\alpha:\beta$  chains underlies the pathophysiology [3]. Both  $\alpha$ -thalassemia and  $\beta$ -thalassemia, however, have a high frequency in many populations; although,  $\beta$ -thalassemia is more prevalent and more widely distributed.  $\beta$ -Thalassaemia is a member of an inherited hemoglobin disorder family that is characterized by reduction of  $\beta$ -globin chain synthesis [9]. The high frequency of thalassemia is due to the protective advantage against malaria that it confers on carriers, analogous to the heterozygote advantage of sickle cell hemoglobin carriers. Thalassemias can result in profound anemia from early life and, if not treated with regular blood transfusions, can lead to death in the first year [7, 10].

### 3. Prevalence and classification of thalassemias

Thalassaemia was originally thought to be a disease limited to the Mediterranean region, in countries such as Greece, Italy, and Cyprus. There is a characteristic distribution of thalassemias in a band around the Old World—in the Mediterranean, the Middle East, and parts of Africa, India, and Asia [4, 5]. As a result, clinically important interactions may occur among different alleles of the same globin gene or among mutant alleles of different globin genes [6, 8]. The prevalence of  $\beta$ -thalassemia trait varies within the Mediterranean coastal regions in Turkey; Mediterranean (Adana 3.7%); Aegean (İzmir 4.8%); and Marmara (İstanbul 4.5%) [4] (**Figure 2**). In highly prevalent regions, an ideal and effective strategy to decrease the birth rate of thalassemia patients is to identify high-risk couples, who are both carriers, before pregnancy by screening (or carrier testing) and then perform a prenatal diagnosis during pregnancy [11].

Thalassemias are genetically classified according to the particular globin chains that are ineffectively synthesized into  $\alpha$ ,  $\beta$ ,  $\delta\beta$ , and  $\epsilon\delta\beta$  thalassemias.  $\alpha$ - and  $\beta$ -thalassemias are important in the public health view [12]. Different hemoglobin mutations have been identified to date, the majority being single nucleotide substitutions, deletions, or insertions of nucleotides leading to a frame shift; rarely does  $\beta$ -thalassaemia result from gene deletions. More than 250 gene defects (more than 1150 mutations have been reported) have been described for different thalassemia phenotypes in different populations [11, 13].  $\beta$ -Thalassemia have been described, and it has been found that the majority thalassemias can be caused by large deletions or by point mutations.  $\alpha$ -thalassemia is often caused by large deletions for single ( $\alpha^+$ -thal) or both  $\alpha$ -genes ( $\alpha^0$ -thal) (**Table 1**) [13].

Thalassemias exhibit a wide spectrum of phenotypes. Depending on the clinical severity, thalassemias are generally divided into three groups: (1) Thalassemia minor/trait: they are carriers who are often asymptomatic and do not need any treatment. (2) Thalassemia intermedia (TI): they have moderate anemia and occasionally require red blood cell transfusion; in  $\alpha$ -thalassemia, it is known as Hb H disease. (3) Thalassemia major (TM): they have severe anemia and require transfusions for survival; in  $\alpha$ -thalassemia, this clinical form was named Hb Bart's hydrops fetalis. The fetus usually dies in utero or shortly after birth [11]. According to the degree of quantitative reduction in the synthesis of normal  $\beta$ -globin,  $\beta$ -thalassemia mutations are classified into three groups: (1)  $\beta^0$ -thalassemia mutation, which results in the absence of  $\beta$ -globin; (2)  $\beta^+$ -thalassemia mutation, which severely reduces the output of  $\beta$ -globin; and

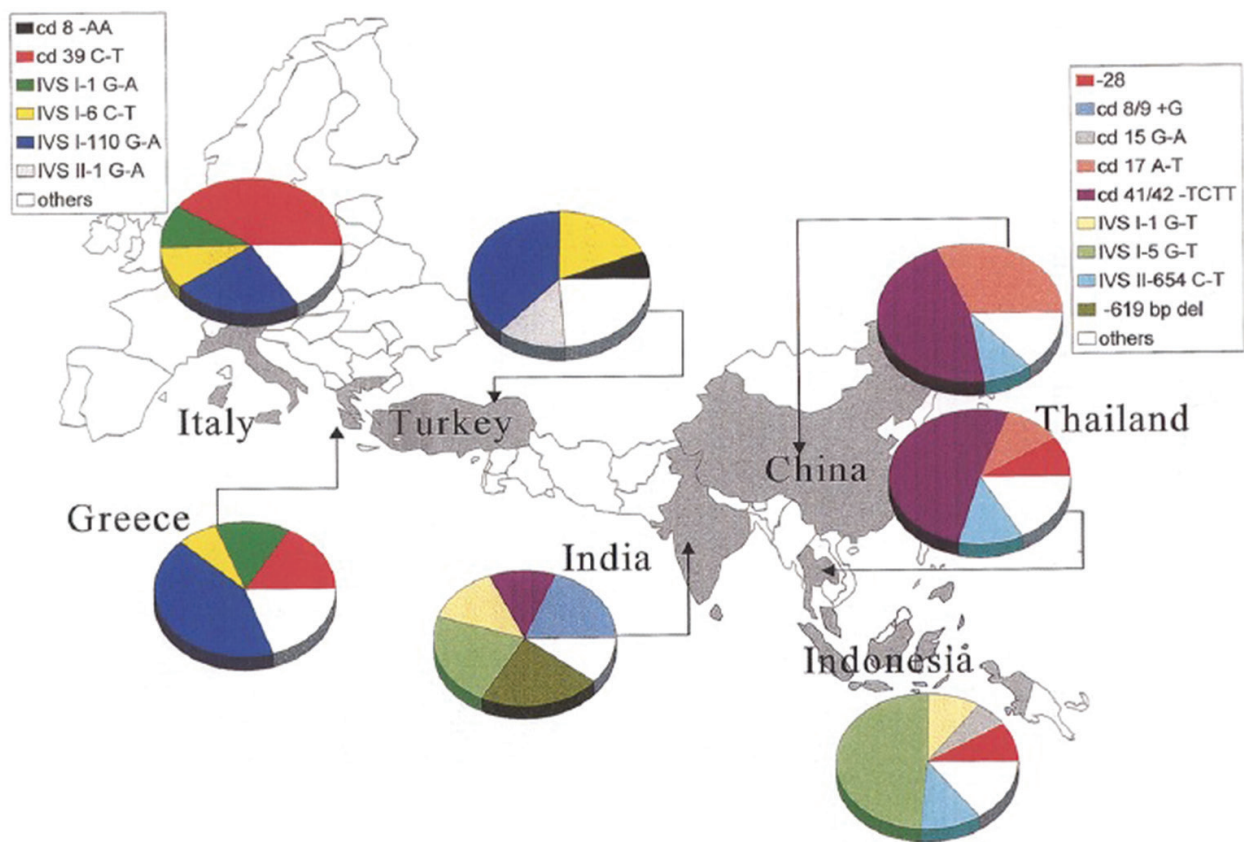


Figure 2. The distribution of common  $\beta$ -thalassemia mutations [7].

Locus	Mutation/deletion types	Common mutations
$\alpha$ -globin	$\alpha^0$ -deletion	-- <sup>SEA</sup> (Southeast Asia), -- <sup>MED</sup> (Mediterranean)
	$\alpha^+$ -deletion	-- <sup>3.7</sup> , -- <sup>4.2</sup> (worldwide)
	$\alpha^T\alpha$ ( $\alpha 2$ gene)	Hb CS (Southeast Asia), $\alpha^{IVS1(-5nt)}\alpha$ (Mediterranean), $\alpha^{PA(AATAAG)}\alpha$ (Middle East Asia)
$\beta$ -globin	$\alpha\alpha^T$ ( $\alpha 1$ gene)	Hb Q-Thailand (Southeast Asia)
	$\beta^{++}$ -mutation	$\beta^{-101(C>T)}$ (Mediterranean)
	$\beta^+$ -mutation	$\beta^{IVS1-110(G>A)}$ (Mediterranean), Hb E (Southeast Asia)
	$\beta^0$ -mutation	$\beta^{CD39(C>T)}$ (Mediterranean), $\beta^{CD41-42(-CTTT)}$ (Southeast Asia)
	deletion ( $\beta$ gene)	619 bp deletion (Asian Indian)
	Deletion (HPFH/ $\zeta\beta$ )	SEA-HPFH, Chinese $\alpha\gamma^+(\Lambda\gamma\delta\beta)^0$ deletion (Chinese)

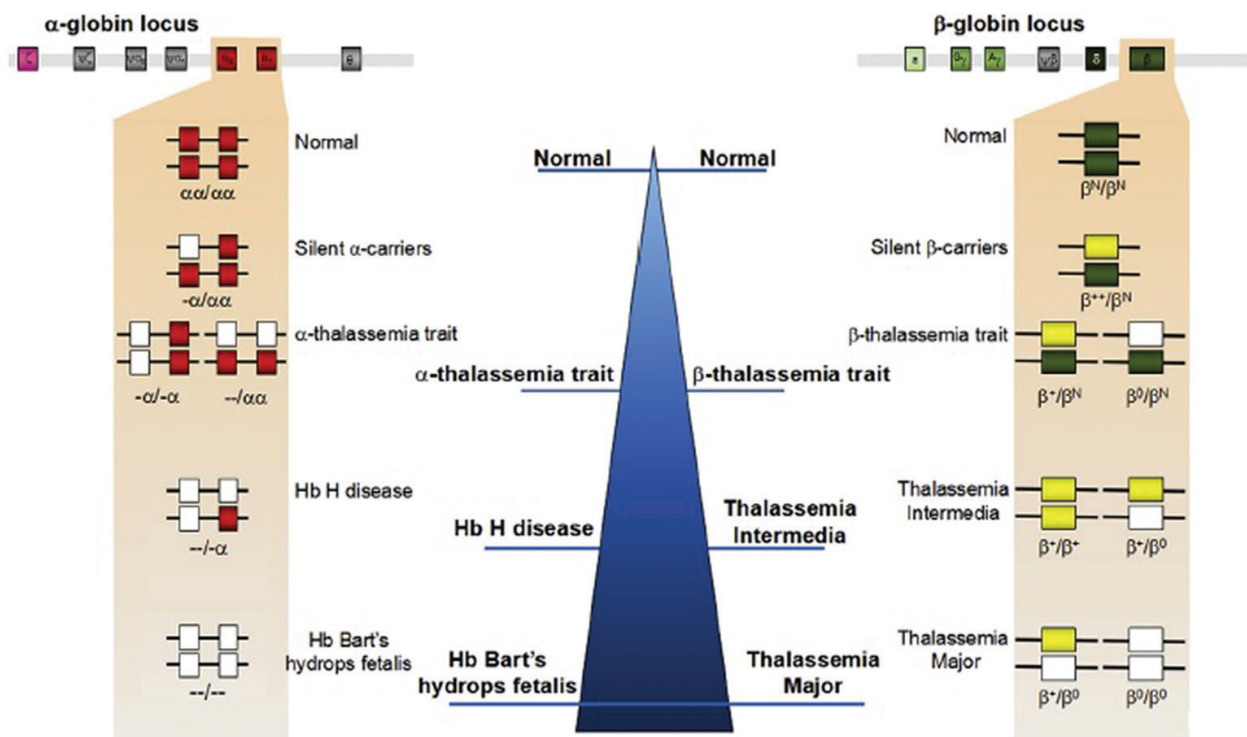
Deletion ( $\beta$  gene): deletions affecting only the  $\beta$ -globin gene.  
Deletion (HPFH/ $\zeta\beta$ ): large deletions involving part or all of the  $\beta$ -globin gene clusters.

Table 1. The common deletions and mutations of thalassemias [13].

(3)  $\beta^{++}$ -thalassemia mutation, also known as silent  $\beta$ -mutation, which mildly reduces the output of  $\beta$ -globin (Figure 3) [11].

Clinical presentation for thalassemia carriers is varied from almost healthy to severe anemia requiring blood transfusions all life [14]. A practical way to prevent thalassemia is identification of carrier couples; genetic counseling and offering prenatal diagnostic services for both carrier couples. Two carrier parents have a 25% chance of having an unaffected child, a 25%





**Figure 3.** Clinic classification and genotype-phenotype association of thalassemias [11].

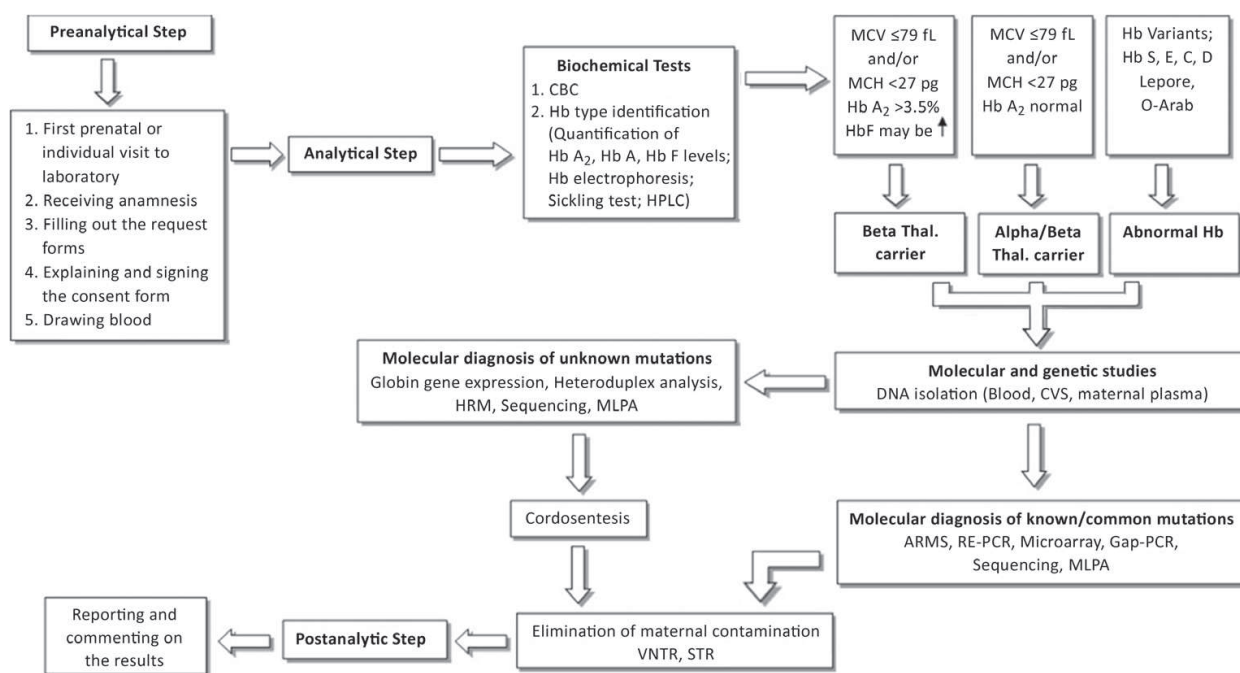
chance of having an affected one, and a 50% risk of having a carrier child. Carriers can only be detected using laboratory methods. Laboratories should increase the diagnostic proficiency for prevention of this blood disorder [13, 15, 16].

In this review, we aim to provide an algorithm consisting of biochemical and molecular methods in the screening of thalassemia carriers and evaluate traditional and new prenatal diagnosis methods for the disorder.

#### 4. Identification of carrier and diseased individuals with thalassemias

Carrier detection is a necessity in populations in which both  $\alpha$ - and  $\beta$ -thalassemia are prevalent. For laboratory diagnosis of thalassemias, molecular diagnostic algorithms should be produced by screening centers (Figure 4). Combining blood hematology/biochemistry and clinical parameters with laboratory analysis, discussing the clinical results with physicians is important as the beginning step for carrier identification. The hematological information obtained from a complete blood count (CBC) is the first essential test for screening thalassemias (Figure 1). Specific mutations of hemoglobin will cause hematologic changes.

Hypochromic microcytic parameters and anemia is generally mild in  $\alpha$ - or  $\beta$ -thalassemia carriers. In cases with rare  $\beta$ -thalassemia, anemia can be intermediate or hemolytic and even severe because it is due to the reduced expression of the thalassemic gene and depends significantly on the degree of  $\beta$ - $\alpha$  imbalance [13].



**Figure 4.** The algorithm of screening and prenatal diagnosis for thalassemias.

The first set of carrier detection procedure is the determination of main erythrocyte indices parameters such as the erythrocyte mean corpuscular volume (MCV), erythrocyte mean corpuscular hemoglobin (MCH) determination and also HbA<sub>2</sub> quantitation. The current screening strategies for thalassaemia carriers are to identify individuals with clinically related mutations. Hypochromic microcytic anemia occurs in both iron deficiency anemia and thalassemias. A decrease in red blood cell count (RBC) and an increase in red cell distribution width (RDW) is accompanied by iron deficiency anemia. Increased RBC count and normal RDW values have been seen in hemoglobin disorders [13]. Serum ferritin levels, the golden standard, will usually be normal or elevated in  $\beta$ -thalassemia carriers but might also be borderline or low in  $\alpha$ -thalassemia carriers [13]. Decreased hemoglobin (Hb) concentration and MCV levels, altered shape and size of the RBCs (anisopoikilocytosis) and existence of target cells are additional hematologic phenotypes observed in individuals with hemoglobin abnormalities. When compared to MCV, the MCH is a more stable parameter, and values of <27 pg and <25 pg have been recommended as alternative screening cut-offs for  $\beta$ -thalassemia and  $\alpha^o$ -thalassemia, respectively (**Table 2**) [17].

#### 4.1. $\beta$ -Thalassemia

Heterozygote  $\beta$ -thalassemia ( $\beta^o$  or  $\beta^+$ ) is characterized by high red blood cell count, microcytosis, hypochromia, increased HbA<sub>2</sub> levels, and unbalanced  $\alpha$ -globin/non- $\alpha$ -globin chain synthesis. Elevation of HbA<sub>2</sub> (standard cut-off value is above 3.5%) is the most important characteristic in identifying heterozygote  $\beta$ -thalassemia [7, 19, 20]. A number of heterozygotes for  $\beta$ -thalassemia may have normal or borderline HbA<sub>2</sub> levels [21]. Some typical carriers have mild  $\beta^+$  thalassemia mutation, i.e., IVSI-6 (T-C) mutation. The  $\delta$ - and  $\beta$ -double heterozygotes exhibit normal HbA<sub>2</sub> level, low MCV and MCH values. These double heterozygote cases should be separated from the  $\alpha$ -thalassemia carriers. Carriers of  $\gamma\delta\beta$ - and  $\delta\beta$ -thalassemias HbA<sub>2</sub> levels are also normal.  $\delta\beta$ -thalassemias have specific elevated HbF

Traits	Hb	Ferritin	MCV	MCH	RBC	RCM
Iron deficiency	↓	↓↓	↓	↓	↓	APC, HY
β-Thalassemia trait	↓	↑↑	↓	↓	↑↑↑	APC, HY, TC
α-Thalassemia (-α/αα)	↓or N	↓N↑	↓	↓	↑	Rare TC, HY
α-Thalassemia (--/αα)	↓	N↑	↓	↓	↑↑	APC, HY, TC, rare IB
α-Thalassemia (-α/-α)	↓	N↑	↓	↓	↑↑	APC,HY, TC
α-Thalassemia (--/-α)	↓	↑↑↑	↓	↓	↓	APC, HY, TC, IB

Hb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; RBC, red blood cell; N, normal; APC, anisopoikilocytosis; HY, hypochromia; TC, target cells; IB, inclusion bodies [18].

**Table 2.** The most significant parameters observed from complete blood count, ferritin, MCV, MCH, RBC, and red cell morphology tests in the common traits at risk of intermediate or severe conditions for thalassemias.

levels, which distinguish this group from the others [5, 12]. In the presence of a normal MCH, hereditary persistence of fetal hemoglobin (HPFH) should be considered. Another problematic group difficult to identify is the silent β-thalassemia and the triple α-gene arrangement. In silent β-thalassemia, the MCV and MCH values are usually normal (i.e., β<sup>+</sup>-101 C > T mutation). The value of HbA<sub>2</sub> and HbF are also normal (**Table 3**) [21, 22].

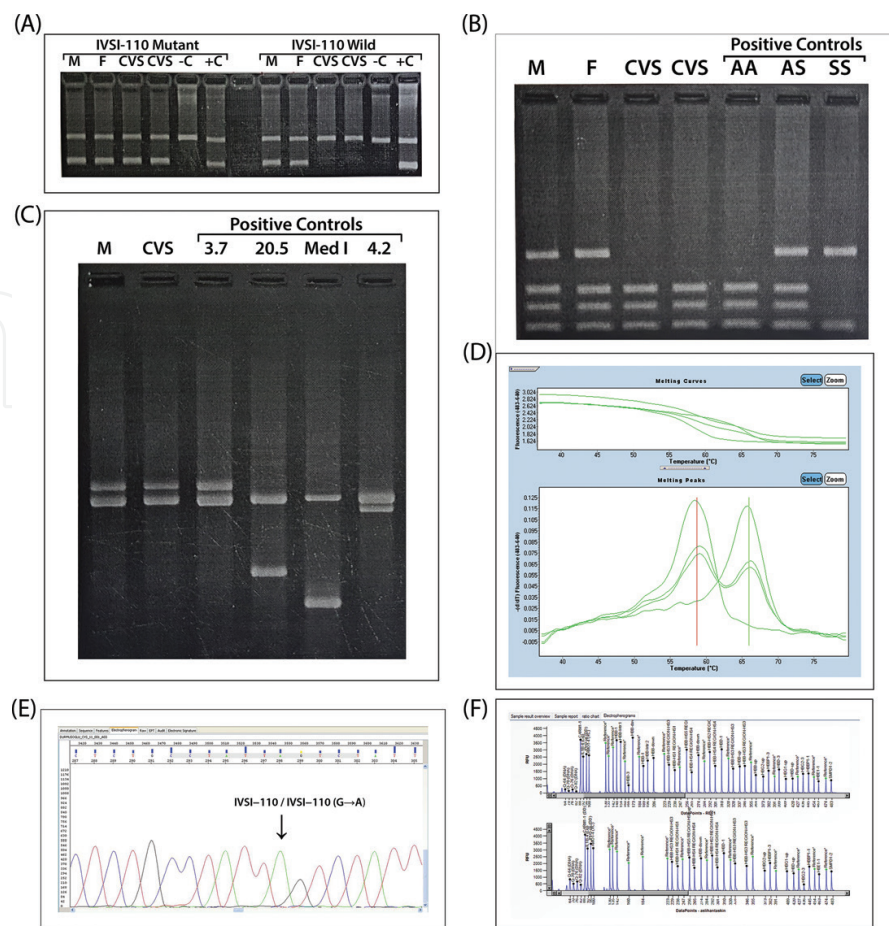
## 4.2. α-Thalassemia

α-Thalassemia mutations are mostly gene deletions. To identify known inherited deletional α-thalassemia mutations, gap-PCR (polymerase chain reaction) is the most common method. A multiplex gap PCR targeting common α gene deletions in a population is most often used as the first step in α-thalassemia genotyping. The sequencing method detects non-deletion α<sup>+</sup>-thalassemia mutations. The five commonest α<sup>o</sup>-thalassaemia deletions can be diagnosed

Phenotype	Genotype
Normal red cell indices	<ul style="list-style-type: none"> <li>• α- and β-thalassemia interaction</li> </ul>
Normal HbA2 level	<ul style="list-style-type: none"> <li>• Iron deficiency</li> <li>• Co-inheritance of δ- and β-thalassemia</li> <li>• Some mild β-thalassemia mutations</li> <li>• γδβ-thalassemia</li> </ul>
Normal red cell indices and HbA2 level (silent)	<ul style="list-style-type: none"> <li>• Silent β-thalassemia mutations</li> <li>• α-globin gene triplication</li> </ul>
Severe heterozygote β-thalassemia	<ul style="list-style-type: none"> <li>• Hyper unstable hemoglobin</li> <li>• Co-inheritance of heterozygote β-thalassemia and triple α-globin gene</li> </ul>

**Table 3.** Interpretations to consider when the hematologic is consistent with atypical β-thalassaemia trait [7].





**Figure 5.** Applications of (A): ARMS-PCR, (B): RE-PCR for, (C): Gap-PCR, (D): HRMA and (E): sequencing analysis, (F): Detection unknown mutations using MLPA for deletional alpha, beta gene mutations (M: Mother, F: Father, C: Control, CVS: Chorionic villus sample).

by gap-PCR: the --SEA allele, the --MED and  $-(\alpha)20.5$  alleles; the --FIL allele, and finally the --THAI allele. The two commonest  $\alpha^+$ -thalassemia deletions are also diagnosed by gap-PCR: the  $-\alpha 3.7$  and  $-\alpha 4.2$  alleles. In our laboratory, the common deletions detected with gap-PCR are  $-\alpha 3.7$ , and  $-(\alpha)20.5$ , --MED and  $-\alpha 4.2$  (Figure 5).

## 5. Molecular methods

Blood count is not always consistent with typical  $\beta$ -thalassemia trait. Furthermore, there is no specific screening test for the clear identification of heterozygote  $\alpha$ -thalassaemia. Only molecular DNA analysis could give certain results in rare mutations [23, 24].

Molecular methods for DNA analysis of hemoglobinopathies currently in use are based on PCR methods that can be used to detect the globin gene mutations. The PCR-based methods differ in identifying hemoglobin variants. Amplification refractory mutation system (ARMS), denaturing gradient gel electrophoresis (DGGE), restriction endonuclease PCR (RE-PCR), real-time PCR, high-resolution melting analysis (HRM), sequencing analysis (Sanger), pyrosequencing,

microarrays can be counted among these PCR-based detecting methods [19, 25]. To detect deletions, Southern blot analysis has been replaced by methods including gap-PCR, multiplex ligation-dependent probe amplification (MLPA), and array comparative genome hybridization (aCGH) [19]. Gene scanning methods such as HRM analysis are also useful for locating possible  $\beta$ -globin gene variants [26]. This technique allows the detection of mutations between the primers used in the assay, which is in contrast to more localized techniques such as hybridization based technologies or restriction enzyme-based assays [27]. In diagnostic use, for this method, it is mandatory to characterize any nucleotide variation by automated sequencing because they do not determine nucleotide changes. Small deletions can be detected by polyacrylamide gel electrophoresis of an amplified beta gene product. Polyacrylamide gel electrophoresis can be used to detect small deletions in an amplified  $\beta$  gene product. Gap-PCR and recently MLPA identifies larger deletions from the  $\beta$ -globin gene [10]. MLPA detects all common, rare, and novel forms of deletional  $\alpha$ -thalassemia (in contrast to gap-PCR) and provides a reliable alternative screening method for the prenatal diagnosis of  $\alpha^o$ -thalassemia [28, 29, 30]. This technique can also identify triple and quadruple  $\alpha$ -gene re-arrangements [31].  $\beta^o$ -Thalassemia, which is caused by small nucleotide deletions and some larger deletions which the whole  $\beta$ -globin gene removes, can be identified by gap-PCR (Hb Lepore, some  $\delta\beta$ -thalassaemia deletions, and the HPFH1/2/3 deletion mutations, etc.).

### 5.1. Sequencing analysis

The parents' DNA whose mutations were not found by classic PCR methods were analyzed by ABI 3130 automatic sequencer. The DNA of the cord blood samples were sequenced to confirm the cordocentesis results. BigDye Terminator v3.1 Cycle Sequencing kit (ABI) and primers that cover all the exons, introns, and exon-intron boundaries of the  $\beta$ -globin gene were used for sequencing analysis.

## 6. Prenatal diagnosis of thalassemias

Prenatal diagnosis created a new option to couples at risk of a major hemoglobinopathy and changed the perspective of screening and counseling for thalassemias [7]. The first step to prevent thalassemia is prenatal diagnosis of these hematological disorders. Prenatal diagnosis for thalassemias is still carried out by traditional conventional methods such as amniocentesis, chorionic villus sampling (CVS), and cordocentesis. These conventional methods have a risk of fetal miscarriage risk around 1% [32].

### 6.1. Blood sampling from parents

Phenotype of parents should be performed by DNA analysis after whole blood count and electrophoresis. Hematology results should be sent to the molecular diagnostic laboratory. Antenatal screening of parents should be performed before the first trimester of pregnancy [17].

## 6.2. Fetal sampling

Fetal DNA for analysis can be obtained by traditional invasive methods; amniocytes, CVS, and cordosentesis [4, 36].

### 6.2.1. Chorionic villus sampling

A fetal-derived tissue, genetically reflecting the fetus and easily accessible in the first trimester (up to 11 weeks) can be used for the prenatal diagnosis of hemoglobinopathies. High-quality DNA can be obtained from CVS material to perform DNA analysis. Maternal contamination is low, especially if careful microscopic dissection is performed to remove contaminating maternal tissues prior to DNA extraction and analysis.

### 6.2.2. Amniocentesis

This is the most commonly used method among invasive prenatal diagnostic methods. Amniotic fluid is the environment of life before the baby is born, and all secretions are in this atmosphere [33]. The prenatal diagnosis result based on an amniocentesis is available later in pregnancy compared to CVS, as amniocentesis is not usually performed earlier than the 15th week [19].

### 6.2.3. Fetal blood sampling

This sampling method can be used for molecular analysis, globin chain synthesis studies, or high-pressure liquid chromatography (HPLC). It can be useful in women at risk of  $\alpha$ -thalassaemia hydrops fetalis. Fetal blood sampling is associated with a higher rate of miscarriage and results are available much later in pregnancy (after 18–20 weeks) [19].

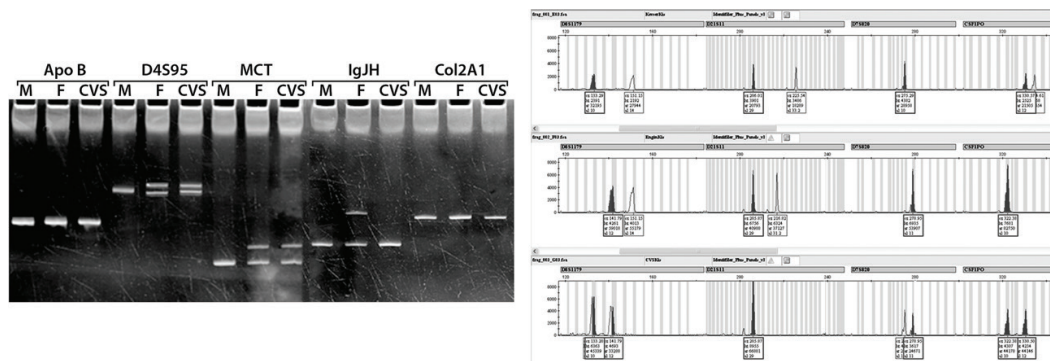
### 6.2.4. HPLC analysis of cord blood

Cord blood is taken in ethylenediaminetetraacetic acid (EDTA) by an obstetrician at 18–20 weeks of gestation. The hemograms are measured for all samples by using an automatic blood cell counter (Coulter T-890). HPLC analysis were studied from the prepared hemoly-sate by Agilent 1100 using the thalassemia short program. The levels of HbA, HbF and Hb Barts, and HbS were estimated.

Molecular diagnostic methods that are based on PCR techniques (ARMS, RFLP, GAP-PCR, VNTR, etc.) and high-throughput technologies (Gene expression, HRMA, microarray, MLPA, NGS, etc.) are currently used in prenatal diagnosis. The molecular genetic tests for prenatal diagnosis within the first 2 months of pregnancy is necessary to prevent infants of carrier couples from being thalassemia [19].

## 6.3. Remove the contamination risk

Maternal cell contamination test is recommended in all prenatal samples to remove the risk of contamination of fetal DNA with maternal DNA. It is important to pay attention to all CVS and AF samples (with or without culture) may have maternal contamination. Maternal contamination should be ruled out after careful dissection of CVS from maternal tissue.



**Figure 6.** VNTR and STR analysis of a family for checking the maternal contamination in CVS. M: Mother, F: Father, CVS: Chorionic Villus Sampling.



**Figure 7.** Noninvasive prenatal diagnosis using cfDNA from maternal plasma.

Polymorphic DNA sites in fetal and parental samples can be identified to monitor maternal contamination (**Figure 6**).

#### 6.4. New diagnostic tests

The discovery of cell-free fetal DNA (cffDNA) in the maternal plasma give the chance to conduct noninvasive prenatal diagnosis (NIPD) during pregnancy [34]. Some encouraging clinical noninvasive approaches to detect paternally inherited mutations such as the detection of fetal sex and RhD status have been improved successfully [35, 36].

Noninvasive tests using cell-free DNA (cfDNA) from a maternal blood sample is also an alternative method, thus eliminating the risk of miscarriage (**Figure 7**). Cell-free fetal DNA (cffDNA) constitutes approximately 10–15% of the total cfDNA and has been shown to represent the entire fetal genome [35].

#### 6.5. Centrifugation

The noninvasive prenatal detection of paternal mutations in  $\beta$ -thalassemias is achievable using HRM analysis, and next generation sequencing of cell-free fetal DNA [36].

### 7. Conclusion

$\beta$ -Thalassemia is an autosomal recessive disorder characterized by hemolytic anemia and microcytosis. It causes decreased synthesis of  $\beta$ -globin chain. This disorder influences 150 million people in large parts of Asia, North Africa, and in the Mediterranean. A wide range of mutations occurs due to the effects of different cultures living together in Turkey. Genetic heterogeneity is more in the southern part of Turkey than in other regions.



Premarital screening and genetic counseling are essential for the prevention and control of thalassemia and hemoglobinopathies [9]. Today, prenatal diagnosis by chorionic villus sampling is an accepted method to protect families having children with thalassemia major and to keep the disease under control. The mutation can be identified from samples obtained by chorionic villus sampling (CVS) and amniocentesis methods by classic polymerase chain reaction (PCR).

The molecular diagnostic algorithms should be produced by genetic diagnosis centers for screening of the carriers and prenatal diagnosis of the couples both of whom carriage has been detected before pregnancy for protection against thalassemia.

## Author details

Abdullah Tuli\* and Ebru Dünder Yenilmez

\*Address all correspondence to: tuliabd@cu.edu.tr

Faculty of Medicine, Department of Medical Biochemistry, Cukurova University, Adana, Turkey

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