

Effects of codon 39 (c>t) mutation on the changes of hematological parameters in children with beta-thalassemia major in the region of Batna, Algeria

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Abstract

Clinical heterogeneity is often due to genetic heterogeneity. One mechanism by which different mutations can produce a phenotypic variation). Beta-thalassemia is mainly caused by a large number of mutations of the gene for beta-globin.

The objective of this study is to correlate molecular lesions and clinical signs in children with beta-thalassemia homozygous. We carried out the molecular genetics of beta globin gene by the method of mini sequencing using Snapshot™ kit (Applied Biosystems) in search of the mutation codon 39 (C>T) (*HBB*: c.118C>T) and we studied haematological parameters by statistical method using the test "t" of Student for comparing the means of the samples. The peripheral blood smear examination was done from the slides stained in Wright stains.

The results obtained have shown that beta-thalassemia homozygous children with severe anemia over 30 ± 0.9 g/L of total Hb with microcytosis of $60,80 \pm 2.014$ fl, hypochromia 18.47 ± 0.6023 pg and the number of red blood cells is 3.220 ± 0.2778 ($10^{12}/L$). In addition, a significantly secondary thrombocytosis and leukocytosis were reported in patients. Thus the electrophoresis of hemoglobin exhibits a high Hb F (55 to 82%). The peripheral smear examination revealed many red cell abnormalities in the blood.

In this study, we used the mini sequencing assay as a rapid screening procedure to identify the severe codon 39 (C>T) mutation in the *HBB* gene. Phenotype of beta thalassemia major is characterized with various hematological parameters.

Keywords: Beta-thalassemia major, Codon 39 (C>T) mutation, *HBB* gene, Hematological parameters, Red cell abnormalities.

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Introduction

Beta thalassemia is a group of inherited autosomal recessive disease characterized by the presence of the defective synthesis chain β -globin part of the hemoglobin molecule, resulting in either partial synthesis (beta plus thalassemia minor; β^+) or complete absence (beta 0 thalassemia major; β^0) [1,2]. Symptoms of beta thalassemia were relatively homogeneous, significant variations in the severity of clinical expression of the disease are observed. The severity depends primarily on the nature of causal mutations in the *HBB* gene but also the association with a variant alpha-globin that will directly influence the balance between the two alpha protein chains and beta globin. It also depends on

the ability of the patient to produce thalassemic fetal Hb in adult life, production will partially or wholly compensate for the lack of adult Hb [3].

In Algeria, the frequency of β -thalassemia gene is 3% [4]; these diseases are a real public health problem often compounded by rate inbreeding of the population (30-32%) [5].

The objective of this study is to correlate very severe molecular lesion (Codon 39 mutation (C>T)) and changes in hematological parameters (Hb, MCV, MCH, red blood cell count, the hemoglobin electrophoresis, and red cell abnormalities) in children with β -thalassemia of homozygous region in Batna, Algeria.

Materials and Methods

Subjects

This study was realized in 7 transfusion dependent β -thalassemia children (beta-thalassemia homozygous) (age brackets 5 to 12 year old of male sex). These subjects are from the region of Batna, cared in the pediatric ward of the University Hospital Batna and we have selected healthy children as a group control.

Blood Collection and Hematological Laboratory Analysis

Venous blood samples of 2.5 ml volume were drawn from the study subjects and were collected in EDTA anticoagulant containers. For these subjects blood withdrawals were performed a few minutes before the regular blood transfusion. The complete blood counts including counts of red blood cell count (RBC), white blood cell (WBC), haematocrit (Hct), platelets (PLT), erythrocyte calculates constants: Mean corpuscular volume (MCV); Mean corpuscular hemoglobin content (MCH) and measurement of hemoglobin (Hb) using aMedonic CA 620-16 hematology analyzer.

HbA2 and HbF values were quantified using electrophoresis before transfusion at alkaline pH by Capillarys[®] PLC (Sebia) while the blood samples were tested in automated cell counter HEMA-TEK 2000 as well as peripheral blood smear examination was done from the slides stained in Wright stains.

Statistical Analysis

The statistical method used is the "t" test of Student for comparing the means of the samples. Results are expressed as mean \pm sem, the calculations were performed using the Graph Pad Prism 5.00 software.

DNA Extraction

The molecular analysis of the *HBB* gene was carried out after taking informed written consent from all the parents of the minors.

Genomic DNA was extracted from peripheral blood leukocytes using the FlexiGene-DNA Kit (Cat # 51206; Qiagen Inc., Valencia, CA, USA) and stored at 4°C.

Minisequencing Reaction of *HBB* Gene

The mini sequencing assay was developed for the detection of the four most common *HBB* genetic variants including three β -thalassemia mutations: codon 39(C>T) (HBB: c.118C>T), IVSI-110(G>A) (HBB: c.93-21G>A) and IVSI-1-2(T>G) (HBB: c.92+2T>G), as well as the hemoglobin S variant (HBB: c.20A>T). To detect these four mutations, an allele specific PCR was performed, followed by highly multiplexed mini sequencing reaction. The specific primer sequences of the *HBB* gene and PCR conditions are available upon request. Polymerase chain reaction products were purified using QIAquick PCR Purification by Kit (Qiagen Inc.). Purified fragments were used as template in a primer extension reaction containing the mutation-specific primer cocktail (Table 1).

Table 1. Primers for multiplex mini sequencing analysis

Investigated mutations*	Minisequencing primers (sequences in 5' >3' direction)
HbS (HBB: c.20A>T)	T(45) ATG GTG CAC CTG ACT CCT G
IVS-I-2 (T>G) (HBB: c.92+2T>G)	T(55) GTG AGG CCC TGG GCA GG
IVS-I-110 (G>A) (HBB: c.93-21G>A)	T(65) ACT GAC TCT CTC TGC CTA TT
Codon 39 (C>T) (HBB: c.118C>T)	T(75) GTG GTC TAC CCT TGG ACC

* The variants are described using Human Genome Variation Society nomenclature

For the extension reaction, we used the SNaPshot Multiplex Kit (Applied Biosystems, Foster City, CA, USA), following manufacturer's instructions. After extension, the samples were treated with shrimp alkaline phosphatase according to the manufacturer protocol.

Multiplex mini sequencing products were resolved by automated capillary electrophoresis ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Briefly, 12 ml of HiDi[™] formamide and 0, 5 ml size GeneScan 120 LIZ-calibrator (Applied Biosystems) were added to 1 ml of multiplex minisequencing product. The mixture was denatured at 95°C for 3 min. next transferred to ice for 2 min. and loaded on an ABI PRISM[®] 310 Genetic Analyzer capillary (Figure 1).

Results

Here we apply the mini sequencing technique as an alternative strategy for genetic diagnosis of *HBB* gene disorders in seven children with β -thalassemia major, provided by the pediatrics department, Hopital Batna (East of Algeria). We chose this method as it allows quick search of the 4 most common and frequent mutations of the *HBB* gene. The GeneScan electropherograms of our patient samples after multiplex mini sequencing primers are shown in Figure 2. In all patients we only found the codon 39(C>T) (HBB: c.118(C>T) mutation in homozygous state. This genetic variant is responsible for the homozygous β -thalassemia phenotype (β^0) [6,7]. The results obtained have shown that beta-thalassemia homozygous children with severe anemia over 30 ± 0.9 g/L of total Hb with microcytosis of $60,80 \pm 2,014$ fl, hypochromia $18,47 \pm 0.6023$ pg and the number of red blood cells is $3,220 \pm 0,2778$ ($10^{12}/L$), WBC= $12,4 \pm 11 \times 10^9/L$, Platelets= $390,9 \pm 141.1 \times 10^9/L$. In addition, hemoglobin electrophoresis shows an increase in fetal hemoglobin (HbF) (55-82%) (Figure 3), the composition of hemoglobin HbA2 is also high (3 to 6%). The peripheral blood smear examination revealed many abnormalities in the blood indices and red blood cells lineage which helped to differentiate thalassemia major. Figure 4 show the marked degree of anisopoikilocytosis is evident with hypochromic microcytic red cells. Target cells and many nucleated red cells are seen (erythroblast basophile and acidophil).

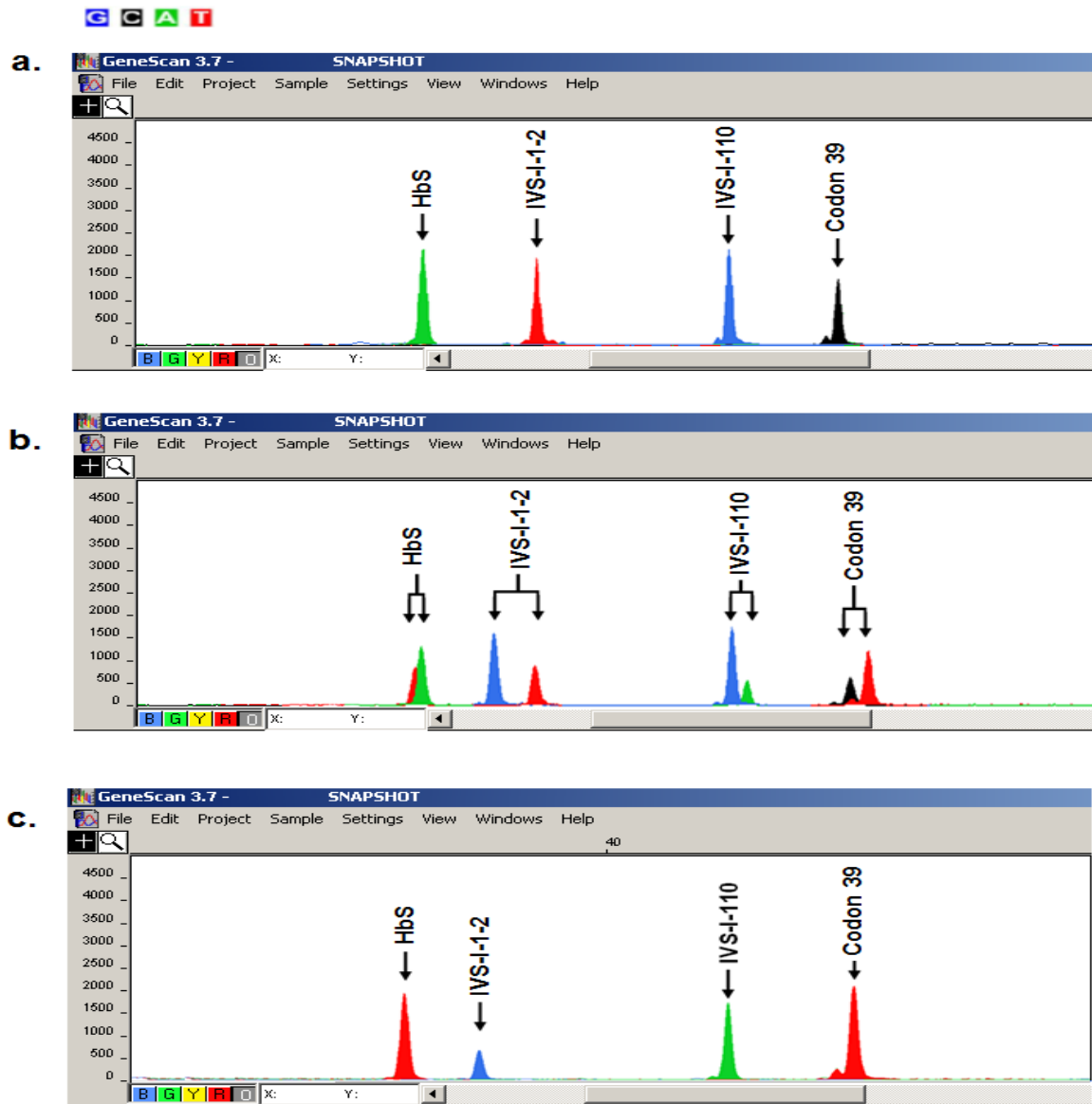


Figure 1. GeneScan analysis of the multiplex mini sequencing reaction. Electropherograms: (a) shows the analysis results of a HBB wild-type sample; (b) illustrates the pattern of peaks for all mutant positions in the heterozygous state; (c) illustrates the pattern of peaks for all mutant positions in the homozygous state

Table 2. Study of hematological parameters of beta-thalassemic children with homozygous codon39 (C>T) mutation

State / Bioassay	Controls	SICK
Hb (g/L)	134,4 ± 0.2410	30±0,9***
RBC (10 ¹² /L)	4.978 ± 0.1077	3,220 ± 0,2778***
HCT %	39,6±2,4	22,31 ±4,3***
MVC (fl)	82.11 ± 2.003	60,80 ± 2,014***
MCH (pg)	29.44 ± 0.6261	18,47 ± 0,6023***
WBC (10 ⁹ /L)	6,9±2,1	12,4±11***
Platelets (10 ⁹ /L)	293±54,1	390,9±141,1***

Note: high significant result (P*** <0.0001)

Discussion

This is the first study investigating the molecular level basis of β-thalassemia in the region of Batna (east of Algeria). We conducted the identification and characterization of the molecular basis of β -thalassemia among children born

in BATNA region. The codon 39 (C>T) mutation was identified by the method of minisequencing multiplex with severe phenotype of beta-thalassemia major with various hematological parameters (Table 2).

The β-thalassemia is characterized by a quantitative reaction, the complete absence of synthesis of the β-globin chain of normal structure: the result of this phenomenon is a decrease, respectively and the absence of functional molecules in the HbA postnatal period and into adulthood ineffective erythropoiesis, typical characteristic of β-thalassemia is the result of many deleterious effects, some of them because of an accumulation of α-globin chains [3,8].

This excess interferes with many normal erythroid maturation stages: some studies in this regard have demonstrated intramedullary death precursors red blood cells, which is accomplished by the arrest in the G1

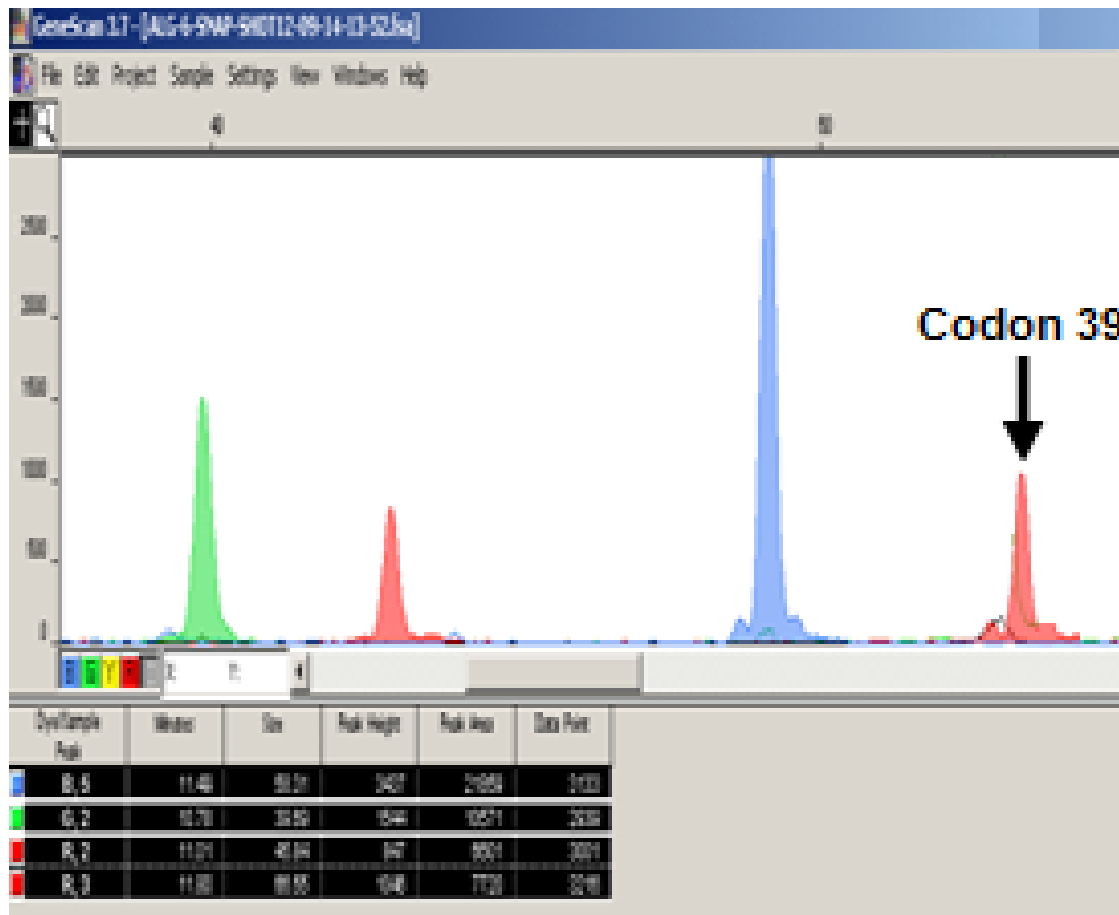


Figure 2. Electropherogram shows a peak for codon 39 (C>T) mutation in the homozygous state

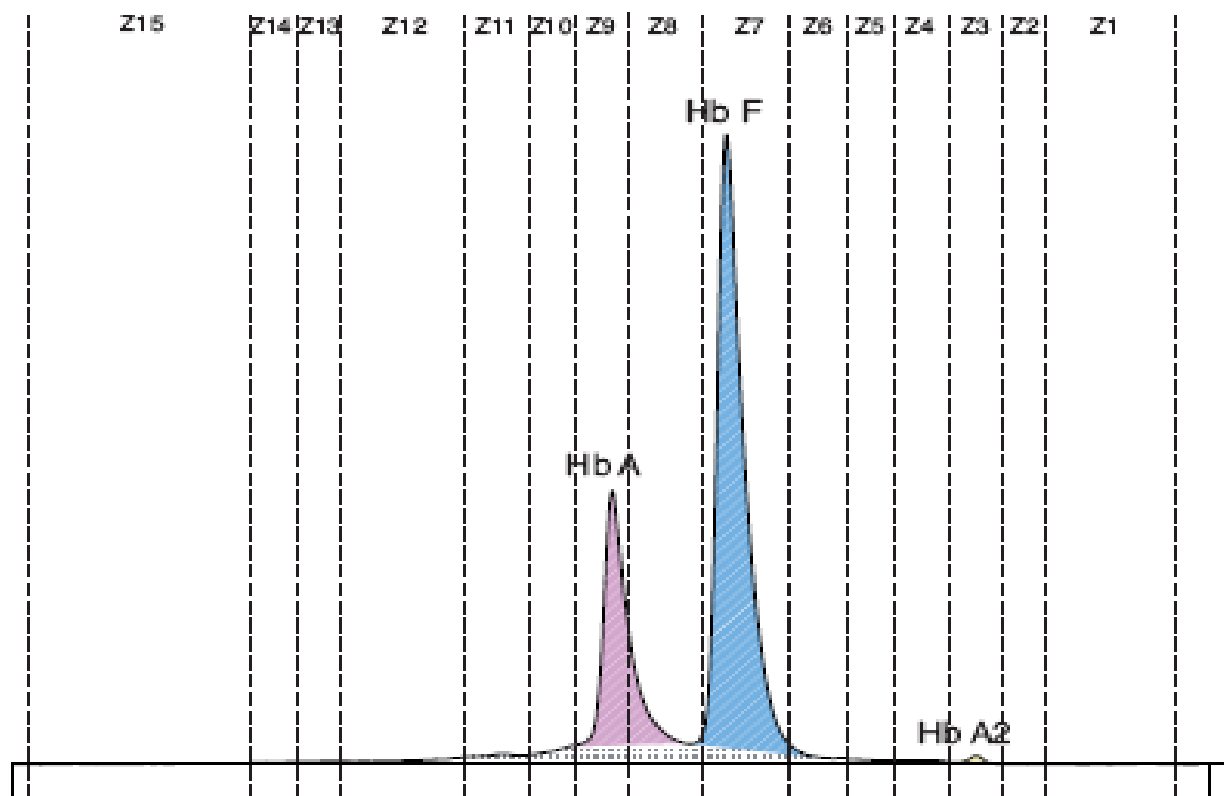


Figure 3. Electrophoretic profile of blood with homozygous beta thalassemia [Hb A (%)=12 à 42%; Hb A2 (%)=3 à 6%; Hb F (%)=55 à 82%]

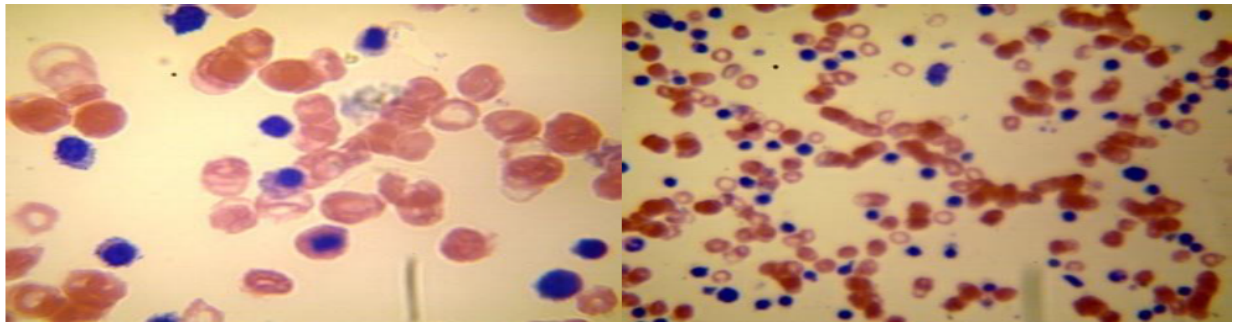


Figure 4. Peripheral blood smear microscopy of a patient with homozygous mutation codon 39 (C>T) mutation. Note: the hypochromia, anisopoikilocytic changes, and nucleated RBC (Wright's stain, 40×100)

phase of the cell cycle and increased spinal erythroblasts from apoptosis formed for the last [9,10]. The clinical manifestations include listless, fatigue, dyspnea, poor appetite, hepatosplenomegaly, heart failure and bone deformation and delayed puberty [11].

Thalassemia is known to be endemic in the Mediterranean area [12]. It is frequent in Algeria and the incidence of the gene is estimated to be between 2 and 3% [13]. It represents a major public health problem, mostly owing to the lack of appropriate transfusion blood and chelators. Previous investigations have disclosed a high molecular heterogeneity of β -thalassemia [14,15].

According the prevalence of β -thalassemia allele in North Africa would increase from west to east Mediterranean countries (Morocco 0, 94%, 1, 4% of Algeria, Tunisia 3% and Egypt 4, 5%) [16-18].

The number of β -thalassemia mutations in Algeria is 25 mutations [12]. On the other hand, the Algerian population is characterized by four dominant mutations, which represent over 80% of β -thalassemia alleles. These are the mutation nonsense codon 39 C→T; IVS-I-110 substitution G→A; frameshift the codon 6 (-A) and mutation IVS-I-1 G→A [19].

The nonsense mutation codon 39 (C>T) is widespread in Algeria with a frequency 25, 94%, 27, 6% [20] and is more common in the west and decreases in center to be predominant in the East [12,20,21].

Patients with thalassemia major have a severe microcytic and hypochromic anemia, associated with increased number of red blood cells and low mean corpuscular volume (MCV) and mean corpuscular Hb (MCH). Peripheral blood smear shows, in addition to microcytosis and hypochromia, anisocytosis, poikilocytosis (spiculated tear drop and elongated cells), and nucleated red blood cells (erythroblasts). The number of erythroblasts is related to the degree of anemia and is markedly increased after splenectomy [22]. The clinical severity of beta-thalassemia is related to the extent of imbalance between the alpha globin and non-alpha globin chains. The non-alpha globin chains include [23].

Conclusion

In this study, we used the mini sequencing assay as a rapid screening procedure to identify the severe codon39 (C>T)

mutation in the *HBB* gene. Phenotype of beta thalassemia major is characterized with various hematological parameters. The genetic diagnosis is designed to confirm the clinical diagnosis and haematological and it will be very important during prenatal diagnosis.

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