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The First Association of Hb Knossos : (*HBB*: c.82G>T) WITH (*HBB*: c.118C>T) Mutation Causs Thalassemia Homozygous in Algerian Children

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Abstract: Beta-thalassemia is the most common disease among hemoglobinopathies in Algeria. Mutations found in Algerian beta-thalassemia patients constitute a heterogeneous group, consisting mostly of point mutations. Only in very rare cases did deletions or insertions cause affected or carrier phenotypes. Hb Knossos (*HBB:* c.82G> T) is a rare variant. In this study, we aimed to investigate the effect of compound heterozygosis for Hb Knossos (*HBB*: c.82G> T) and (*HBB*: c.118C>T). To our knowledge, this is the first report of such a combination related with beta-thalassemia major phenotype in a Algerian family, we used the minisequencing assay as a rapid screening procedure to identify most common *HBB* genetic variants and direct DNA sequencing to detect the rare mutations of *HBB* gene. Heterozygous inheritance of the mutation results in severe beta-thalassemia phenotype. The proband was a 13-year-old boy when first studied. He was referred because of severe anemia. Hematological analysis of the reveals Hb 7.2 g / dl; with microcytosis of 71.1 fl, hypochromia 25 pg and the number of red blood cells is 2.9 106 / mm³ . In addition, a significantly secondary thrombocytosis and leukocytosis were reported in patient. Electrophoresis of hemoglobin in an alkaline medium shows Hb A2 = 4% HbF = 65% and blood smear confirms microcytosis hypochromia, and showing the presence of many dacryocyte with hyper eosinophilia. **[The combination of these mutations Hb Knossos (*HBB*: c.82G> T) and (*HBB:* c.118C>T) causes the beta-thalassemia major phenotype, and this is important for genetic counseling**.*Biomedicine and Nursing* 2021;7(1):71-76**]**. ISSN 2379-8211 (print); ISSN 2379-8203 (online). http://www.nbmedicine.org. 11. doi:10.7537/marsbnj070121.11.

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INTRODUCTION

β-thalassemia is a recessive monogenic disorder encountered worldwide with a higher prevalence among Mediterranean, Middle Eastern and Indian populations (Weatherall DJ, 1991). The disease is due to mutation in b globin locus for which more than 200 alleles have been reported (Weatherall DJ and Clegg JB 2001).

In Algeria, the frequency of β-thalassemia gene is 3% (Belhani M , 2009); these diseases are a real public health problem often compounded by rate inbreeding of the population (30-32%) (Bennani C *et al*, 1994). Previous investigations have disclosed a high molecular heterogeneity of β -thalassemia (Bennani C *et al,* 1993).

In this study, we describe a rare hemoglobin variant caused by a mutation in β--globin gene, HBB:c.82G[T: Codon 27 GCCTCC (Ala/Ser), Hb Knossos, which produces the classical phenotype of homozygous β -thalassemia in association with *HBB*: c.118C>T) mutation causes thalassemia homozygous in an Algerian children.

Hemoglobin (Hb) Knossos is a silent β-thalassemia variant, which was first described in a Greek family and later was detected in other families of Mediterranean origin (Fessas PH *et al* , 1982; Baklouti F *et al* 1986).

It was demonstrated that some *B*- Knossos RNA transcripts utilize a cryptic splice sequence and are therefore abnormally processed (Orkin SH *et al,* 1984) and the amount of the variant in carriers is approximately 30 % of the total hemoglobin (Arous N *et al* , 1982). In heterozygous state, the mutation results in mild bthalassemia phenotype with a normal HbA2 level, whereas the homozygous state results in intermediate b-thalassemia (Baklouti F *et al* , 19986; Sahli CA *et al* , 2012).

Here we describe the molecular and hematological characterization of first observation of association of Hb Knossos: HBB: c.82G>T with

HBB: c.118C>T) mutation wich causes thalassemia homozygous in an Algerian children.

MATERIALS AND METHODS

The variant was first found in a 13-year-old Algerian boy presented as a homozygous thalassemia syndrome, with anemia (Hb 7, 2 g/dl). The biochemical and hematological values, as well as the red cell morphology, were consistent with homozygous beta--thalassemia, except for an unusually low level of Hb F (about 65%). He was born to non consanguineous parents. This patient is from the region of Batna in northeast Algeria, cared in the pediatric ward of the University Hospital Batna. Laboratory diagnosis is based on two main and reproducible tests:

Hematological Tests:

Blood count with red blood cell count (RBC) , white blood cell (WBC), platelets (PLT) ,erythrocyte calculates constants: Mean corpuscular volume (MCV); Mean corpuscular hemoglobin content (MCH) and measurement of hemoglobin (Hb) using a Medonic CA 620-16 hematology analyzer. Haematological and biochemical parameters were measured before any transfusion

Biochemical Tests:

Electrophoresis hemoglobin Capillarys 2 (sebia):

The CAPILLARYS HEMOGLOBIN (E) kit enables, on CAPILLARYS instruments, the efficient separation of hemoglobin fractions and detection of a large number of hemoglobin variants and thalassemias patterns.

The CAPILLARYS HEMOGLOBIN (E) assay is based on the principle of capillary electrophoresis in free solution. Hemoglobin fractions are separated in silica capillaries, by their electrophoretic mobility and electroosmotic flow at a high voltage in an alkaline buffer. Hemoglobin fractions are directly detected at the specific absorbance of 415 nm.

The Peripheral Blood Smears Examination:

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.

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 minisequencing 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 minisequencing 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 (see Table 1).

Table 1. Primers for multiplex minisequencing 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 minisequencing 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.

Sequencing:

The β -*globin* gene was amplified using couples of primers: *HBB* F: 5'- CTG ACA CAA CTG TGT TCA CT-3'and *HBB* R: 5'- TTC ACC TTA GGG TTG CCC -3'.

The β - thalassemia mutation was identified by automated sequence analysis performed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the fl uorescent dideoxy-termination method (Big Dye-Terminator Cycle Sequencing Kit, Applied Biosystems, Foster City, CA, USA).

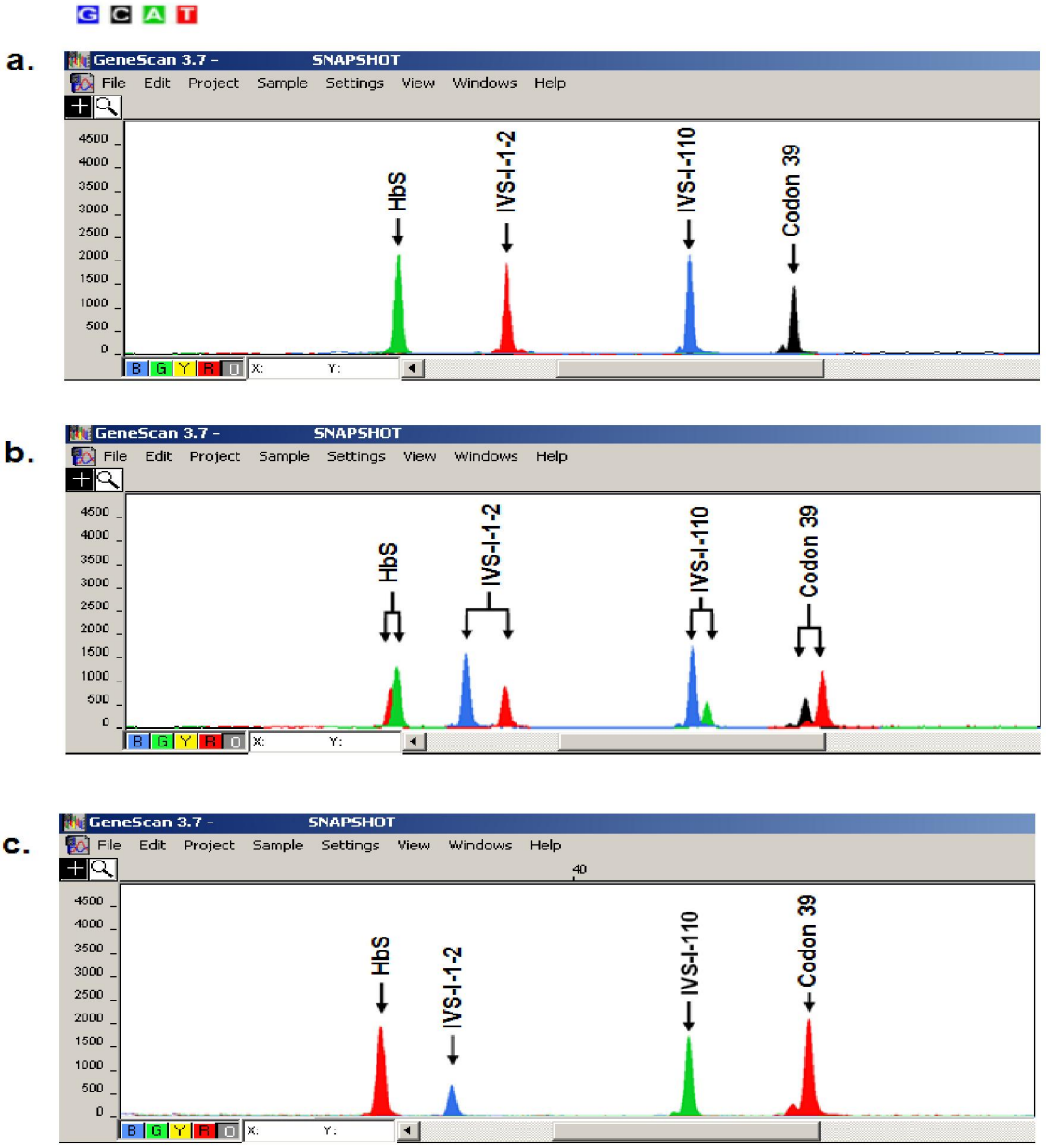

Figure1. GeneScan analysis of the multiplex minisequencing reaction. Electropherograms: (a) shows the analysis results of a *HBB* wild-type sample; (b) illustrates the pattern of peaks for all mutant positions in the heterozygous state; (c) illustrates the pattern of peaks for all mutant positions in the homozygous state.

RESULTS AND DISCUSSION

We found this variant of hemoglobin in association with codon 39 (C> T) mutation in a patient from Batna region. The proband was a 13-year-old boy, when he was studying for the first time. He was referred because of hypochromic microcytic anemia. Hematological analysis reveals Hb 7.2 g / dl; the number of red blood cells is 2.9 106 / mm³. WB = 6.6 × 10³ / mm³, PLT = 226 × 10³ / mm³, VGM = 71.1 µl, TCMH = 25 pg. The electrophoresis of hemoglobin in an alkaline medium shows Hb A2 = 4% HbF = 65% and the blood smear confirms microcytosis hypochromia, and showing the presence of numerous dacryocytes with hyper eosinophilia. No abnormal Hb was detected by electrophoresis Capillarys in alkaline medium.

Molecular exploration allowed us to identify the presence of two different mutations on both alleles. We concluded that this child is doubly heterozygous for a genotype. DNA analysis by the HBB gene minsequencing method revealed codon 39 mutation (C> T) and direct DNA sequencing revealed a second mutation at codon 27 of exon 1 with GCC> TCC (HBB: c.82G> T) is the substitution of G for T at nucleotide position 82 (figure 2). This change was identified as HBB: c.82G> T, known as Hb Knossos in the HbVar database (figure 3).

This hemoglobin has also been discovered in the heterozygous state in two Algerian families (1983; Morlé *et al.,* 1984), and a family from West Indies India (Galacteros *et al.,* 1984). The first homozygous case of Hb Knossos was reported in northeastern Algeria (Baklouti *et al.,* 1986). Among the Arab countries, Hb Knossos was later identified in Egypt (Olds *et al.,* 1991), Tunisia, Jordan, and Gaza with a frequency varying between 0.1% and 3.3% of total alleles (Zahed, 2001)

According to (Phaedon *et al.,* 1986), it becomes certain that the Hb Knossos anomaly causes a relatively modest syndrome of intermediate β-thalassemia when associated with a classical β-thalassemia gene. Hemoglobin Hb Knossos, due to the interaction of this mutation with a β-globin gene. However, patients are in good clinical condition despite their extremely low hemoglobin levels (usually 6-7 g / dl). They proposed that the low oxygen affinity of Hb Knossos contributes significantly to the mildness of the clinical picture (Papasotiriou *et al.,* 1983); A similar indication also derives from the study of Hb Knossos / Hb Lepore heterozygous compounds (Morlé *et al.,* 1984). The hematological parameters are: marked hypochromic anemia with very microcytic indices. These alterations are confirmed by red blood cells with morphology characterized by striking poikilocytosis and microcytosis; they give the impression of being caused by extensive fragmentation of the erythrocytes. Reticulocytes are only slightly increased or not at all associated with hemoglobin deficiency (Phaedon *et al.,* 1986).

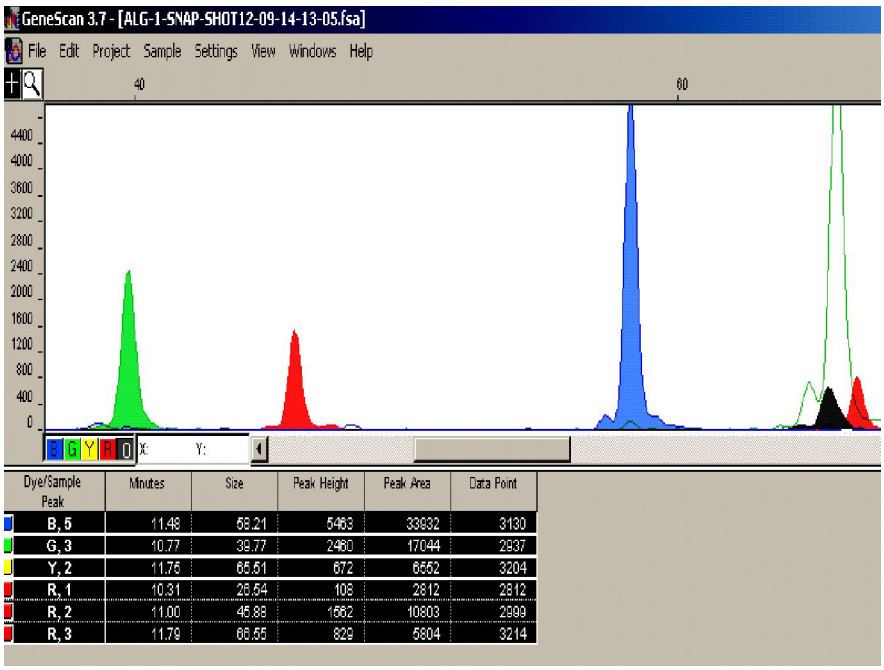

Figure 2. Electropherogram shows a peak for codon 39(C>T) mutation in the heterozygous state.

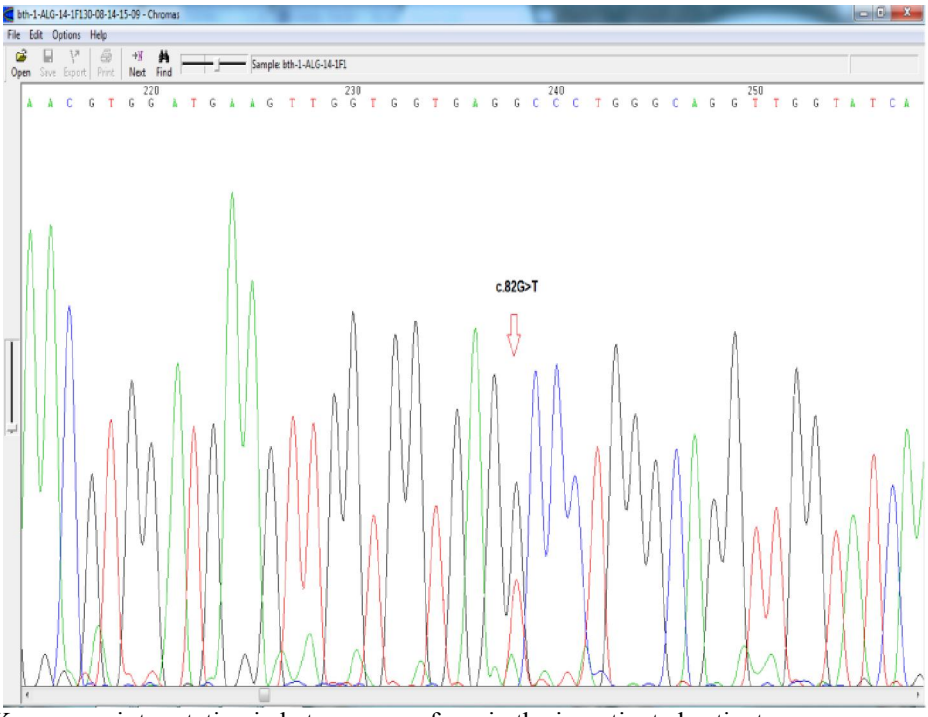

Figure 3. Hb Knossos point mutation in heterozygous form in the investigated patient

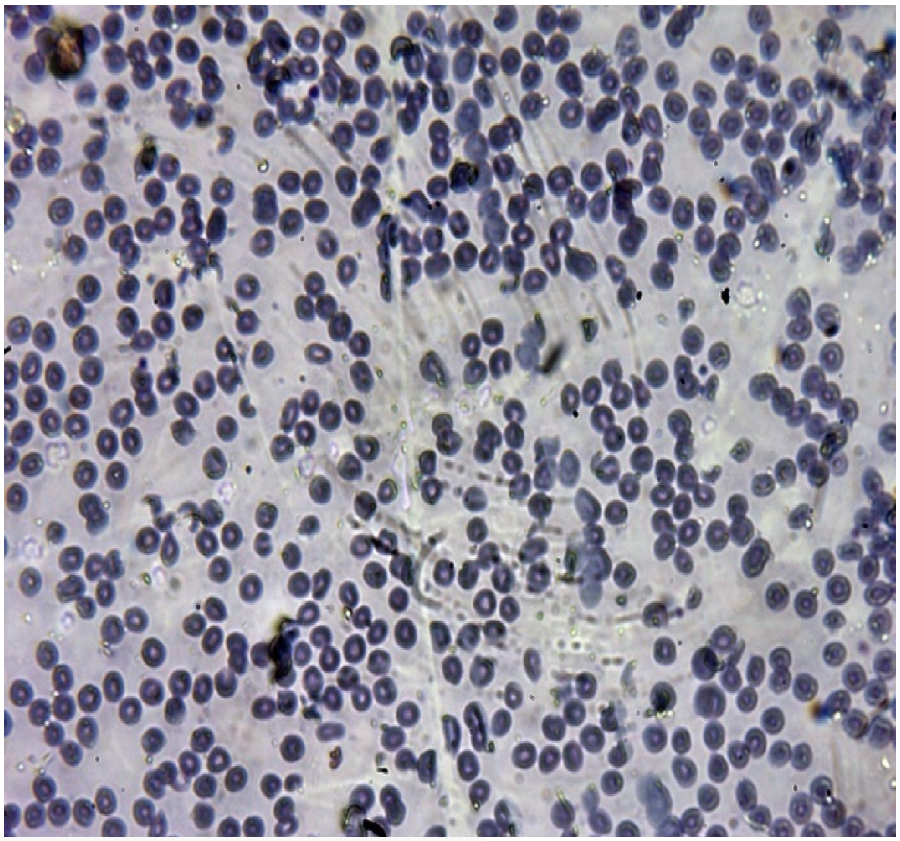

Figure 4. Peripheral blood smear microscopy of a patient shows a combination of Hb Knossos with codon 39 mutation (C> T) showing microcytosis hypochromia, a presence of many dacryocytes with hyper eosinophilia. (Wright coloring: 40 × 100).

CONCLUSION

In this study, we used the minisequencing assay as a rapid screening procedure to identifie 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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