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### ► To cite this version:

Azam Amirian, Morteza Karimipoor, Masoumeh Jafarinejad, Maryam Taghavi, Alireza Kordafshari, et al.. First report on the co-inheritance of beta-globin IVS-I-5 (G→C) thalassemia with delta globin CD12 Asn→Lys (AAT→AAA)HbA -NYU in Iran.. Archives of Iranian Medicine, 2011, 14 (1), pp.8-11. pasteur-00829009

**HAL Id: pasteur-00829009**

**<https://riip.hal.science/pasteur-00829009>**

Submitted on 1 Jun 2013

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## Original Article

# First Report on the Co-inheritance of Beta-globin IVS-I-5 (G $\rightarrow$ C) Thalassemia with Delta Globin CD12 {Asn $\rightarrow$ Lys (AAT $\rightarrow$ AAA)} HbA<sub>2</sub>- NYU in Iran

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## Abstract

**Background:** Co-inheritance of  $\beta$ - and  $\delta$ -globin mutations in Iran is not uncommon. This situation may interfere with correct diagnosis and genetic counseling of  $\alpha$ - and  $\beta$ -thalassemia in screening programs. Here we report the co-inheritance of  $\beta$ - and  $\delta$ -globin gene mutations in an individual with microcytosis, hypochromia and a normal hemoglobin A<sub>2</sub> (HbA<sub>2</sub>) level.

**Methods:** Genomic DNA extraction, amplification refractory mutation system (ARMS) polymerase chain reaction and direct DNA sequencing of  $\delta$ - and  $\beta$ -globin genes were exploited for detection of the mutations in these two genes in an individual with low hematological indices and normal HbA<sub>2</sub>.

**Results:** ARMS-PCR technique revealed the  $\beta^+$  IVSI-5 (G to C) mutation and direct DNA sequencing of the  $\delta$ -globin gene detected a previously reported delta codon 12 (AAT $\rightarrow$ AAA) HbA<sub>2</sub>-NYU. This study reports HbA<sub>2</sub>-NYU in association with the  $\beta$  IVSI-5 (G to C) mutation in Iran.

**Discussion:** This report emphasizes that normal HbA<sub>2</sub> expression in a  $\beta$ -globin carrier is due to mutation in the  $\delta$ -globin gene and may cause misdiagnosis of thalassemia.

**Keywords:**  $\beta$ -thalassemia,  $\delta$ -globin, HbA<sub>2</sub>-NYU, Iran

## Introduction

$\beta$ -thalassemia is an autosomal recessive disorder. Patients who inherit two  $\beta$ -thalassemia mutations are usually transfusion dependent  $\beta$ -thalassemia major and eventually have to use lifelong iron chelation therapy to combat the tendency for iron overload.<sup>1</sup> Patients who inherit milder mutations may have  $\beta$ -thalassemia intermedia and  $\beta$ -thalassemia heterozygotes are normal or mildly anemic, with no clinical symptoms.<sup>2</sup> There is a delicate balance between production of globin chains in normal erythropoiesis. Ineffective erythropoiesis, shortening of the erythrocyte life span and clinical expression of  $\beta$ -thalassemia are all related to the degree of chain imbalance.<sup>3</sup> Thus far over 200  $\beta$ -globin mutations have been reported, which usually lead to either a reduction or absence of  $\beta$ -globin chain production.<sup>4,5</sup> In many countries in the Mediterranean area, thalassemia is a major health problem, the same is true in Iran located in the southwest of Asia, especially in northern and southern parts of the country.<sup>6</sup> A premarital screening

program for prevention of major  $\beta$ -thalassemia has been in effect since 1997.<sup>7</sup>

Some of the  $\beta$ -thalassemia phenotypic variations can be accounted for by interaction with different numbers of  $\alpha$ - or  $\delta$ -globin genes. The  $\delta$  locus determines the  $\delta$ , or nonalpha chain of HbA<sub>2</sub> ( $\alpha$ -2/ $\delta$ -2). The mutations of the  $\delta$ -globin gene that solely decrease the quality or quantity of  $\gamma$ -globin chain synthesis have no clinical effect. The non-alpha chains, designated  $\delta$ -chain, differ from the  $\beta$ -chain in 10 amino acid residues.<sup>8</sup> Although  $\delta$ -globin gene (HBD MIM#142000) mutations have no clinical implications, the co-inheritance of  $\beta$ - and  $\delta$ -thalassemia may lead to misdiagnosis because HbA<sub>2</sub> levels remain normal or low due to decreased  $\delta$ -chain production. For this reason, the detection of  $\delta$ -globin alleles is important in countries that have implemented a thalassemia prevention program because of a high incidence of  $\beta$ -thalassemia carriers. Several variants of the  $\delta$ -chain resulting from single amino acid substitutions have been described in Sicily.<sup>9</sup> HbA<sub>2</sub>-NYU HBD c.39T>A is one  $\delta$ -chain variant that is the fifth  $\delta$ -chain variant resulting from the substitution of lysine for asparagine at the exon 1 of  $\delta$  12, which was found in an Iranian family residing in Tabriz.

The identification of factors that modulate the expression of the disease is relevant to our understanding of pathophysiology and genetic counseling. IVSI-5 (G $\rightarrow$ C) is the most common mutation reported in the southern part of

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Accepted for publication: 30 June 2010

the country. Here we report the molecular findings of an individual with microcytosis, hypochromia, and a normal HbA<sub>2</sub> level. Our findings showed that he had an IVSI-5 (G→C) mutation along with the HbA<sub>2</sub>-NYU mutation in his δ-globin gene.

## Patients and Methods

In laboratory investigations of the families who were referred to our center as a part of the National Program for Prevention of Thalassemia in Iran, a family from Tabriz was also referred for carrier detection and prenatal diagnosis. The proband was a 28-year-old married male with no children, residing in Golpayegan in northwestern Iran. He was a known case of hypochromic microcytic anemia with a normal HbA<sub>2</sub>, and a higher RBC level in comparison to his wife. Although the RBC level is slightly higher in males, many other factors such as smoking and hypo-hydration might have led to this increased RBC level. The proband's Fe, ferritin, and total iron binding capacity (TIBC) levels were within normal ranges (Table 1).

### DNA analysis

After obtaining informed consent, blood samples (10 mL) were collected in tubes that contained EDTA. A total of 2 mL of each sample was used for complete blood count and Hb electrophoresis. Complete blood count was performed using a Sysmex automated cell counter (Sysmex Kx-21, Germany), according to the manufacturer's recommendation. For Hb electrophoresis, erythrocyte lysates were analyzed by cellulose acetate electrophoresis.<sup>10</sup> The lysates were also applied to column chromatography to quantitate HbA<sub>2</sub> levels.

DNA was extracted from blood samples (5 mL) using the

salting out method.<sup>11</sup> The amplification refractory mutation system polymerase chain reaction (ARMS-PCR) was exploited for detection of β-globin gene mutations.<sup>12</sup> Primers were selected from Weatherall and Clegg.<sup>13</sup> For ARMS PCR, the thermal cycling regimen consisted of 27 cycles: preheating at 94°C/min, annealing at 68°C/min, extension at 72°C/2 min and post-extension at 72°C/4 min. The amplification reaction was performed in a MyCycler™ thermal cycler (Biorad, USA). A total of 10 μL of the PCR products were loaded on a 1.5% agarose gel and the amplicons were visualized by UV transillumination after staining with ethidium bromide. The α-globin gene analysis was performed by multiplex Gap-PCR and direct DNA sequencing.<sup>14</sup>

DNA sequencing was performed on an ABI 3130 Genetic Analyzer (Kawsar Biotech Co., Iran). For this, two specific primer sets were designed. The first set amplified a 947 bp fragment on the δ-globin gene that included exon I, exon II and their flanking regions on the δ-globin gene. The sequences of these primers were: δ1F 5'-CTG AGT CAA GAC ACA CAT GAC AG -3' and δ1R 5'-TGG TAT GCA TAA TTT GAG TTG TTG -3'. The second primer set amplified a 1133 bp fragment which included exon III and its flanking regions on the δ-globin gene. The sequences of these primers were: δ2F 5'-AAT ATC CTG TCT TTC TCT CCC AAC -3' and δ2R 5'-TAA TTT CTG CTC TTT GGA GGT AG -3'. The primers were manufactured by Prim, Italy (ch 11: nt 55454 to nt 56540 of accession No.U01317).

## Results

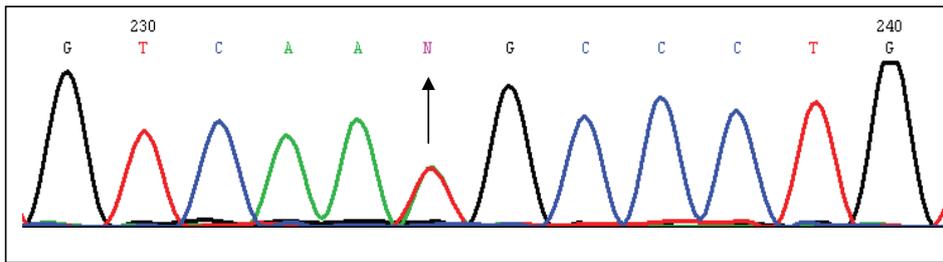
Here we report the result of molecular studies from an individual referred for molecular characterization of the β-globin gene as a part of the National Premarital Screening

**Table 1.** Hematological data of a family with the combination of β-thalassemia/HbA<sub>2</sub>-NYU

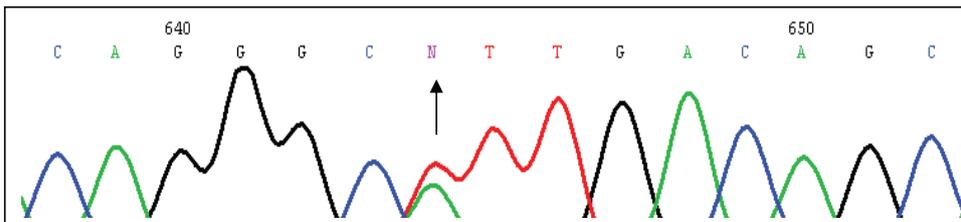
	Proband	Spouse	Proband's father	Proband's mother
Found mutation	δ CD12(AAT→AAA) & IVSI-5(G→C)	-5nt(-TGAGG)	δCD12(AAT>AAA)	IVSI-5(G→C)
Sex-age	28	27	58	50
Ferretin ng/mL)	118	49	—	—
Iron (ug/dL)	63	56	—	—
TIBC (ug/dL)	265	251	—	—
Hb (g/dL)	12.6	11.9	14.5	—
RBC (10 <sup>12</sup> /L)	6.99	4.86	5.5	5.56
MCV (fL)	58.9	75.5	83.1	63
MCH (pg)	18.0	24.5	26.2	19.9
MCHC (g/dL)	30.6	32.4	32	31.2
HbA (%)	94.4	97.4	97.8	94.6
HbA <sub>2</sub> (%)	2.0	2.1	1.6	4.8
HbF (%)	0.6	0.5	0.6	0.6
Hb Var (%)	3.0	0.0	3.0	0.0

TIBC= total iron binding capacity

## Forward graph



## Reverse graph



**Figure 1.** Direct sequence analysis results show the 947 bp fragment of the  $\delta$ -globin gene. The T $\rightarrow$ A substitution at the third base of codon 12 is indicated by the letter N (up: forward, down: reverse).

Program for  $\beta$ -thalassemia. During Hb electrophoresis analysis we observed normal  $A_2$  (2.0), and decreased MCV and MCH. Molecular analysis of the proband showed that his mutation in the  $\beta$ -globin gene was IVSI-5 (G $\rightarrow$ C). Direct DNA sequencing of the  $\beta$ -globin gene also confirmed heterozygous status for the IVSI-5 (G $\rightarrow$ C) mutation with two neutral polymorphisms, one in the second intervening sequence (IVS-II), IVSII-666 (T $\rightarrow$ C) and another at codon 2 (CAC $\rightarrow$ CAT). In this case, one would expect to see elevated HbA<sub>2</sub>. In order to determine the molecular basis for this case we decided to investigate the status of the  $\delta$ -globin gene. Restriction fragment length polymorphism (RFLP) analysis of the  $\delta$ - and  $\beta$ -globin regions indicated no deletion (data not shown). We then analyzed the DNA sequence of the  $\delta$ -globin gene. Sequence analysis revealed a  $\delta$ -CD12 [AAT $\rightarrow$ AAA, (most probably represents) Asn $\rightarrow$ Lys] HbA<sub>2</sub>-NYU mutation in the heterozygous state both in the proband (Figure 1) and his father. Since the HbA<sub>2</sub>-NYU mutation and the  $\beta^+$  IVS-I-5(G $\rightarrow$ C) mutations were observed in the father and mother of the proband, respectively, therefore these mutations in the proband could have not been segregated in a single chromosome. Thus these mutations are in the *trans* state. Also, attempts to locate any mutations in the  $\alpha$ -globin genes of the proband by direct sequencing and multiplex-PCR were unsuccessful.

The hematological and hemoglobin analysis data for HbA<sub>2</sub>-NYU mutation presents normal indices, except for the reduced HbA<sub>2</sub> level.

## Discussion

Cases with low MCV and MCH, and normal HbA<sub>2</sub> levels

are due to various reasons (e.g.,  $\alpha$ -globin carrier status,  $\delta/\beta$  gene deletion, other forms of globin-gene cluster deletions, etc.). Molecular characterization of underlying causes of abnormal cases is very important. Failure to do so may cause potential pitfalls in genetic counseling and prenatal diagnosis. This is true in countries like Iran where thalassemia and its underlying molecular causes are prevalent and heterogeneous from one region to another. It is especially important where diverse ethnic groups are to be investigated or an extensive prevention program is underway to offer the choice of prenatal diagnosis (PND) for at-risk couples and thereby reduce the incidence of births affected with severe forms of hemoglobinopathies.

In an earlier report that has described two Russian-Jewish families, equal proportions of HbA<sub>2</sub> and NYU (both hemoglobins 1.3%) were encountered,<sup>15</sup> while in our report the relative amount of the variant was more than HbA<sub>2</sub>. HbA<sub>2</sub> was 2.0% and HbA<sub>2</sub>-NYU, 3.0% (Table 1). Another previous study in Sicily has shown mean values of normal and variant HbA<sub>2</sub> at 1.5% $\pm$ 0.2 and 1.6% $\pm$ 0.2, respectively.<sup>9</sup> In this study, they detected ten subjects that were heterozygous for HbA<sub>2</sub>-NYU. Three were associated with a -100 CAP polymorphism. In one case, HbA<sub>2</sub>-NYU was linked with the -100 Cap polymorphism,  $-\alpha^{3,7}$  and Hb Valletta (HBB c.262A $\rightarrow$ C,  $\beta$  cd87 ACA $\rightarrow$ CCA). In that case, levels of HbA<sub>2</sub> and HbA<sub>2</sub>-NYU were similar to those detected in the simple heterozygous state for HbA<sub>2</sub>-NYU (HbA<sub>2</sub>, 1.6%; HbA<sub>2</sub>-NYU, 1.8%). That study suggested borderline levels of HbA<sub>2</sub> in the presence of severe  $\beta^+$ - or  $\beta^0$ -thalassemia defects and normal levels of HbA<sub>2</sub> with mild  $\beta^+$ -thalassemia defects such as IVSI-6 (T $\rightarrow$ C),<sup>9</sup> while the present study has shown a normal level of HbA<sub>2</sub> in the presence of severe  $\beta^+$  IVSI-5(G $\rightarrow$ C).

Simple carriers of  $\delta$ -globin gene mutations are completely normal and not at reproductive risk for severe hemoglobinopathy syndromes. The potential problem occurs for individuals who carry both  $\beta$ -thalassemia and the  $\delta$ -globin gene mutation, since they will not have an elevated HbA<sub>2</sub> and may be mistaken for  $\alpha$ -thalassemia or iron deficiency.<sup>16</sup> There are several earlier reports on the co-inheritance of different  $\delta$ -globin gene mutations with  $\beta$ -globin gene mutations in other populations.<sup>17</sup> Our case is the first report on the co-inheritance of a  $\delta$  CD12 (AAT $\rightarrow$ AAA, Asn $\rightarrow$ Lys) with the  $\beta^+$  IVSI-5(G $\rightarrow$ C) mutation in Iran. This case and our recent reports on the co-inheritance of HbA<sub>2</sub>-Troodos with the IVSII-I (G $\rightarrow$ A)  $\beta^0$ -thalassemia<sup>18</sup> or HbA<sub>2</sub>-Etolia with the IVSI-I (G $\rightarrow$ T)  $\beta^0$ -thalassemia,<sup>19</sup> may suggest that the frequencies of the  $\delta$ -chain variant might be high in the Iranian population. It can also be suggested that different forms of  $\delta$ -globin mutations are present as well. Thus it suggests that  $\delta$ -globin gene mutations are common in Iran, raising the possibility of misdiagnosis of  $\beta$ -thalassemia carriers. In cases with HbA<sub>2</sub> variants, it is important to consider the Hb electrophoresis carefully in order to avoid an incorrect diagnosis concerning  $\beta$ -thalassemia. Moreover, this report suggests that a study for  $\delta$ -globin gene defects should be considered as a step in the detection of at-risk couples in our region. Also, the possible influences of these globin gene defects in other populations could be better explored.

## Acknowledgements

This research was supported by the Pasteur Institute of Iran. We would like to thank the patients for their cooperation. We are also grateful to the authorities of Golpayegan, Tabriz, and Farmanfarmaian Health Care Centers for their cooperation and referral of the individuals to our laboratory.

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