

## Brief Report

## Co-inheritance of Hemoglobin D and $\beta$ -thalassemia Traits in Three Iranian Families: Clinical Relevance

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### Abstract

Here we report the result of three cases referred to our lab that had a combination of  $\beta$ -thalassemia and hemoglobin D (Hb D) traits. These individuals had no symptoms of profound anemia and hematological indices were similar to that of a  $\beta$ -thalassemia heterozygote. In all three cases, the Hb D level was elevated and no HbA was detected electrophoretically. The electrophoresis pattern suggested that all cases were homozygotes for Hb D. PCR followed by digestion with *EcoRI* and sequencing of the  $\beta$ -globin gene confirmed the presence of Cd 121 GAA>CAA in the heterozygous form with another  $\beta$ -globin mutation. In all cases, the mutations in the  $\beta$ -globin gene were detected by ARMS PCR technique and they were either IVSII-I or IVSI-5. Hematological studies of the family members showed that thalassemia which caused the mutations and Hb D were in the *trans* position.

**Key words:** ARMS-PCR,  $\beta$ -thalassemia, HbD, heterozygous, Iran

### Introduction

Thalassemias are among the most common genetic disorders worldwide, occurring more frequently in the Mediterranean region and Southeast Asia.<sup>1</sup> The disease causes anemia of variable severity depending on the type of disease and the causative mutation.<sup>2</sup>  $\beta$ -thalassemia is the most prevalent genetic disorder in our region.<sup>3</sup> Approximately 180 different mutations affecting the  $\beta$ -globin gene lead to reduction or absence of  $\beta$ -globin chain production, resulting in -thalassemia.<sup>4</sup>

As with numerous other countries in the Mediterranean region, thalassemia is a major health problem in Iran where it is more frequently seen in the northern and southern regions of the country.<sup>5</sup> In Iran, a premarital screening program for the prevention of major  $\beta$ -thalassemia has been in effect since 1997. Prior to marriage, couples are tested to determine if they are carriers of the  $\beta$ -globin gene mutation or not. For those who are carriers, prenatal diagnosis is offered.<sup>3,6</sup>

Hemoglobin D (Hb D), a hemoglobin variant, occurs mainly in north-west India, Pakistan and Iran.<sup>3</sup> Hb D differs structurally from normal hemoglobin A (HbA) at the amino acid 121 position on the  $\beta$  chain, where glutamine replaces glutamic acid.<sup>1</sup>

There are several Hb D variants, amongst them Hb D-Punjab (also known as Hb D-Los Angeles) is by far the most common.<sup>7,8</sup> Hb D can occur in at least four forms: heterozygous Hb D trait, Hb D-thalassemia (Hb D/ $\beta^0$  or  $\beta^+$ ), Hb D/Hb S and homozygous Hb D

Two main differential diagnoses of homozygous Hb D are HbD/ $\beta$  and Hb D/S disease. These two conditions should be differentiated by using the parameters such as red cell indices, HbA<sub>2</sub> and HbF levels, and family studies.<sup>9</sup> Numerous reports have shown that individuals who have co-inheritance of Hb D and  $\beta$ -globin mutations are clinically asymptomatic. Hb D is probably the most common Hb variant in Iran (personal observations).

Hb D-Punjab results from a single base substitution at codon 121 (GAA>CAA) of the  $\beta$ -globin gene. Here we report three families who co-inherited Hb D and  $\beta$ -globin gene mutations and the impact on Hb electrophoresis. Families were referred to our laboratory at Pasteur Institute of Iran, as this Institute is the National Reference Center for Prenatal Diagnosis (PND) of thalassemia and hemoglobinopathies.

### Materials and Methods

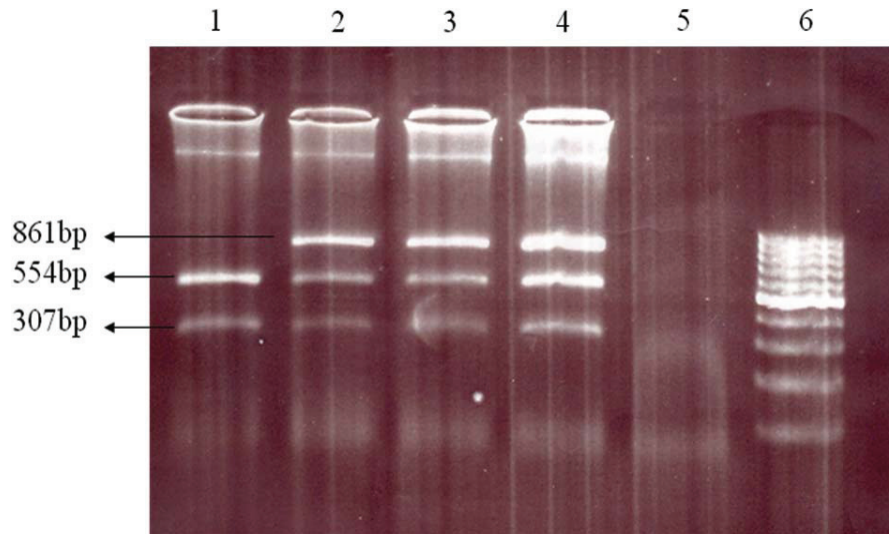
#### Subjects

During laboratory investigations on families referred to us as a result of the National Program for Prevention of Thalassemia in Iran,<sup>6</sup> three families were referred to our lab for further analysis. In the first family, the proband was a 29 year-old male from Ardebil, a northern city of Iran. The second family was from Kerman, a southern province, and the third case was a 31-year-old male from Khuzestan, a western province. All subjects had low MCV, MCH and high HbA<sub>2</sub>, with no normal HbA levels. All had no symp-

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**Figure 1.** The PCR product (861 bp) digested by *ECOR1* enzyme. Lane1: Normal, Lane 2: Heterozygous control (-/+), Lanes 3, 4: Two propositus, Lane 5: Blank, Lane 6:100bp ladder

**Table 1.** Laboratory data of three families with a combination of  $\beta$ -thalassemia/Hb D

	Age	Hb (g/L)	RBC ( $10^{12}/L$ )	HCT (%)	MCV (fL)	MCH (pg)	RDW	Hb A (%)	Hb A <sub>2</sub> (%)	Hb F (%)	Hb D (%)
Propositus 1	29	12.6	6.7	41.2	61.3	18.8	20.8	0.0	5.6	0.2	94.2
Father 1	—	12.5	6.33	39.5	62	19.7	—	93.7	6	0.3	—
Mother 1	—	12.6	4.39	35.5	81	26.8	—	57	2	0.4	40.6
Propositus 2	—	14.8	6.40	44.1	68.9	23.1	—	0.0	4.0	5.0	91
Wife 2	—	13.0	4.70	39.0	83.0	27.7	—	96.6	2.8	0.6	—
Son 2	31	12.9	6.2	41.3	66.4	20.7	16.0	93.7	4.9	1.4	—
Propositus 3	31	14.6	7.7	46.7	60.6	18.9	19.8	0.0	5.5	0.0	94.5

Hb=hemoglobin, RBC=red blood cells, HCT=hematocrit, MCV=mean cell volume, MCH=mean cell hemoglobin, RDW=red cell distribution width

toms of profound anemia or any history of blood transfusions (Table 1).

#### DNA Analysis

After obtaining informed consents, blood samples were collected in tubes containing EDTA and transported to the laboratory for analyses. Two ml of each sample were used for complete blood count and Hb electrophoresis. Complete blood count was performed by Sysmex automated cell counter (Sysmex Kx-21 Germany), as recommended by the manufacturer. For Hb electrophoresis, erythrocyte lysates were analyzed by cellulose acetate electrophoresis. The lysates were also applied to column chromatography in order to quantitate HbA<sub>2</sub> levels.

DNA was extracted from the blood samples (5 mL) with the salting out method.<sup>10</sup> Amplification refractory mutation system PCR (ARMS-PCR) was used for the detection of common  $\beta$ -globin gene mutations in Iran.<sup>11</sup> Primers were selected from a published report.<sup>12</sup> For ARMS PCR, the thermal cycling regimen consisted of: 27 cycles, preheating at 94°C for 4 minutes, denaturing at 94°C for 1 minute, annealing at 67°C for 30 seconds and extension at 72°C for

1.5 minutes. The amplification reaction was performed in a MyCycler™ thermal cycler (BioRad, USA). A total of 10  $\mu$ L of the PCR products were loaded on a 1.5% agarose gel and the amplicons were visualized under UV transillumination after staining with ethidium bromide. For molecular characterization of Hb D (codon 121), a region containing exon 3 of  $\beta$ -globin was amplified with the following primers:

RE (5'-CAATGTATCATGCCTCTTTGCACC-3') and RD (5'-GAGTCAAGGCTGAGAGATGCAGGA-3'). The 861 bp PCR product of this amplicon was digested by EcoRI restriction enzyme (Roche, Germany), according to manufacturer recommendations. In order to rule out other nucleotide changes in the  $\beta$ -globin gene and confirm the results; direct  $\beta$ -globin gene sequencing was performed in both directions, on an ABI3130 Genetic Analyzer (KBC, Iran).

#### Results

Here we report the results of hematological and molecular studies from three individuals referred to our lab for mo-

lecular characterization of the  $\beta$ -globin gene as a part of the National Premarital Screening Program for  $\beta$ -thalassemia carriers.<sup>3</sup> For all three cases, in addition to low MCV and MCH levels in their complete blood counts, elevated HbA<sub>2</sub> (4 – 5.6%) and an extra abnormal Hb peak (91 – 94%) in the Hb D/G/S position, with near zero Hb A was noted by Hb electrophoresis (Table 1).

PCR-RFLP showed that the three probands are carriers of Hb D (heterozygous). In Hb electrophoresis, the level of Hb D was greater than 91% and the mutation in the  $\beta$ -globin gene was IVSII-I (G to A), as confirmed by  $\beta$ -globin gene sequencing.

Molecular analysis of the probands showed that the mutations in their  $\beta$ -globin genes were IVSIIInt 1(G to A) (two subjects) and IVSI nt 5 (C to G), respectively. In all three cases, the other nucleotide change was Cd121 GAA>GCC (known as Hb D-Punjab). Family studies indicated that the mothers of the first and third proband were heterozygous for Hb D. In the second case the father was a carrier of Hb D. In all cases, the other parent was a carrier of the  $\beta$ -globin mutation.

## Discussion

Laboratory data suggested that in the first family, the propositus father was responsible for transmitting the defective  $\beta$ -globin gene to his son, while the mother proved to be the source of the Hb D allele. Indeed her Hb D level in electrophoresis was (40.6%). This showed that IVSII-1 and Hb D (CD121) were co-inherited from each parent and the two alleles were in the *trans* position. The high level of Hb D (95%) suggested that the propositus is homozygous for Hb D, however molecular studies confirmed that he is heterozygous for this variant, which is the case in every family studied. In these cases, it seems that the  $\beta$ -globin chain is produced from a chromosome that carries both Hb D variant and the other alleles which are responsible for the mutation (IVSII-1 and IVSI-5) without production, therefore, in Hb electrophoresis, no normal Hb A was detected. This report emphasizes the importance of careful molecular analysis in Iranian premarital  $\beta$ -thalassemia screening programs so that ambiguities in Hb electrophoresis can be resolved.

As indicated, none of the individuals in this report had profound anemia and their clinical presentations resembled  $\beta$ -thalassemia heterozygotes. However, some cases have been reported that have compound heterozygosity of Hb D/ $\beta$ -thalassemia who occasionally needed blood

transfusions.<sup>13</sup> To date, after screening more than 8000  $\beta$ -thalassemia carriers, of which some were also Hb D carriers, we have not seen HbD/ $\beta$ -thalassemia carriers who required blood transfusions or were profoundly anemic.

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