

**Original Contribution****HB STARA ZAGORA: A NEW HYPER-UNSTABLE HAEMOGLOBIN CAUSING SEVERE HAEMOLYTIC ANAEMIA****G. Petkov<sup>1</sup>, L. Simjanovska<sup>2</sup>, P. Chakarova<sup>1</sup> and G. Efremov<sup>2</sup>**<sup>1</sup>Paediatric Clinic, Faculty of Medicine, Stara Zagora, Bulgaria;<sup>2</sup>Research Centre for Genetic Engineering and Biotechnology, Macedonian Academy of Sciences and Arts, Skopje, Republic of Macedonia**ABSTRACT**

We describe a new hyper-unstable  $\beta$ -chain variant (codons 137-139, -6 bp) in a 2-year-old Bulgarian boy. The abnormal haemoglobin is associated with severe haemolytic anaemia as a consequence of its hyper-instability. The child was admitted to the Paediatric Clinic at the age of two months. Because of anaemia (Hb 6.9 g/dl) and high serum iron level (58 $\mu$ M/l) the child was transfused. However, a month later his haemoglobin dropped to 7.5 g/dl, and since then the child is once a month on regular blood transfusion. Haemoglobin analysis of a blood sample collected two months after the last transfusion at the age of 2 years revealed no abnormalities, except the presence of inclusion bodies after incubation of peripheral blood with brilliant cresyl blue. Sequencing of the  $\beta$ -globin gene revealed heterozygosity for a 6 bp deletion ((- TGGCTA) at codons 137 (the 2<sup>nd</sup> and the 3<sup>rd</sup> bp), 138 and 139 (the 1<sup>st</sup> bp) forming a new codon at position 137 (GAT). This event eliminates three amino acids (Val-Ala-Asn) and introduces a new residue (Asp). It creates a new restriction site for Hph I. The patient and his twin brother had no history of haemolysis. The paternity of the child was confirmed by DNA analysis.

**Key Words:** unstable; Hb; St.Zagora; de novo.**INTRODUCTION**

Since the first description of dominantly inherited  $\beta$ -thalassaemia (1, 2) more than 30 families/cases with this condition have been described (3-5) They are caused by mis-sense mutations, minor deletions, and frameshifts arising from minor insertions and deletions, which result in elongated or truncated  $\beta$ -chain. Unlike the recessively inherited  $\beta$ -thalassaemia, most of the dominantly inherited  $\beta$ -thalassaemia mutations result in the synthesis of  $\beta$ -chain variants which are very unstable.

In this report we describe a new mutation in exon 3 of the  $\beta$ -globin gene, arising as a de novo mutation, in a Bulgarian boy who developed hemolytic anemia at 2 months of age. The mutation is deletion of 6 bp at codons 137-139 leading to an abnormal  $\beta$ -chain 144 amino acids long. This highly unstable variant is termed Hb Stara Zagora after the geographic location in which the patient reside.

**PATIENT AND METHODS**

Red blood cell (RBC) indices were determined

with a Coulter Counter model S (Coulter, Hialeah, FL). Routine haematological studies were performed with standard procedures. Methodology to analyse blood samples for the presence of inclusion bodies and unstable Hb and for the relative synthesis of  $\alpha$  and  $\beta$  chains has been presented before (6). The presence of a possible abnormal Hb in red cell lysates was evaluated using starch gel electrophoresis (7) and starch gel electrophoresis in the presence of urea (8) and polyacrylamide gel electrophoresis (PAGE) in the presence of urea-acetic acid-Triton X-100 (9); and chromatographic procedures routinely in use in our laboratory (10). Hb F was quantified by alkali denaturation (11) and Hb A<sub>2</sub> was determined by DE-52 microcolumn chromatography (12) and HPLC (10).

DNA was isolated from WBC by the procedure described by Poncz et al (13). Haplotyping was done for four members of the family and involved the following restriction sites: HincII 5' to  $\epsilon$ , HindIII at  $\epsilon$ <sup>G</sup> $\gamma$  and  $\epsilon$ <sup>A</sup> $\gamma$ , Hindi at  $\Psi\beta$  and 3' to it, Avall at  $\beta$ , and BamHI 3' to  $\beta$ . Methodology and probes have been listed before (14, 15). The  $\alpha$ -globin gene number was determined by gene mapping with methodology routinely in use in our

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laboratory(10, 16). DNA sequence analysis was performed on ABI PRISM™ 310 automated sequenator using Big Dye Terminator v1.1 Cycle Sequencing kit (PE Applied BioSystems, Foster City, CA, USA). Fragment of 632 bp containing exon 3 of the β-globin gene was PCR amplified using the following primers:

- Forward - 5'ATACAATGTATCATGCCTCTTTGC ACC 3';
- Reverse - 5'GTATTTTCCCAAGGTTTGAAGCTAG CTC 3'. 20 mL of PCR amplified DNA was digested overnight with *Hph I* restriction enzyme (New England Bio Labs, Beverly, MA, USA). The digested fragments were analysed on 10 % PAGE.

RNA analysis was performed as described earlier (17, 18). Total cellular RNA was isolated from the reticulocyte-enriched top fraction obtained by centrifugation of 5 ml fresh blood by the procedure of Chomczynski and Sacchi<sup>19</sup>. Briefly, about 1 µg of RNA was reverse transcribed (RT) using 50 pM of specific reverse primer (5TCAAGGCCCTTCATAATATCCCCCA 3') from the 3' untranslated region of the β globin gene. The RT product was next subjected to PCR using 100 pM of forward primer from the beginning of exon 3 of the β globin gene (5'ACCCACCCAGTGCAGGCTG3') and 50 pM of the reverse primer used for RT. The following PCR program was used: 10 min of initial denaturation at 95°C, 25 cycles of 1 min denaturation at 95°C, 1 min of annealing at 56°C and 1 min of extension at 72°C. PCR was ended for 10 min of extension at 72°C.

The RT/PCR products were separated on 8 % PAGE.

Paternity was confirmed by analysis of 15 STR polymorphic loci (D8S1179; D21S11; D7S820; CSF1PO; D3S1358;TH1; D13S317; D16S539; D2S1338; D19S433; vWA; TPOX; D18S51; D5S818; FGA and the Amelogenin locus using AmpFI STR-Identification Kit, analysed on ABI Prism 310 Sequenator (PE Applied BioSystems, Foster City, CA, USA).

Protein structural analyses were made on tryptic peptides of the β chain, which was isolated from whole red cell lysate of the propositus by carboxymethyl (CM)-cellulose chromatography. The methodology included the separation of peptides by HPLC procedures and amino acid analysis using fully automated equipment. Technical details of these methods have been published (6).

**RESULTS**

The propositus was 2 months old when referred to the Department of Paediatrics. He was born with twin brother from first gestation. His mother developed mild anaemia during the pregnancy, which was treated with iron therapy. At the age of 2 months, when visiting the Department of Paediatrics for consultation, anaemia was noticed. His Hb was 6.9 g/dl and the serum iron 58 µM/l (twice above the reference value). Because of the low Hb concentration a transfusion was given. A month later the Hb level was again low (7.5 g/dl) and since then red cell transfusions were necessary every 4 weeks.

The blood counts and haematological parameters are summarised on **Table 1**.

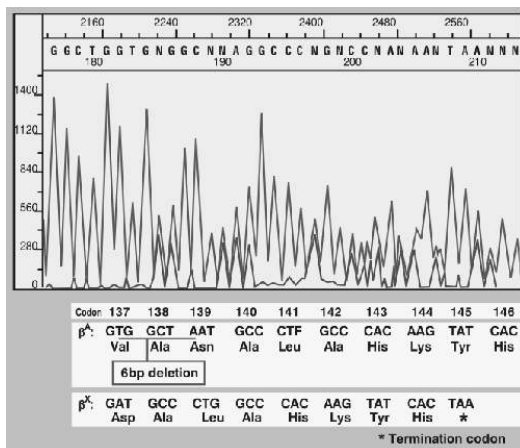
**Table 1.**

	Proband			Twin brother	Mother	Father
Age/yr	1	2	3	3	34	30
Hb (g/dl)	5.9	7.1	6.0	12.0	11.9	14.2
RBC (10 <sup>12</sup> /L)	2.1	2.5	2.0	4.4	4.1	4.3
PCV (L/L)	0.18	0.24	0.16	0.34	0.33	0.38
MCV (fL)	84	96	81	76	81	88
MCH (pg)	28.0	28.2	30.0	27.1	28.9	33.0
Reticulocytes (%)	10.5	9.7	9.4	1.8	1.7	2.0
Morphology	Abnormal*	Abnormal*	Abnormal*	Normal	Normal	Normal
Heinz Bodies	++	++	++	-	-	-
Stability test	-	-	-	-	-	-
Starch gel electrophoresis	AA <sub>2</sub>	AA <sub>2</sub>	AA <sub>2</sub>	AA <sub>2</sub>	AA <sub>2</sub>	AA <sub>2</sub>
PAGE	α <sup>A</sup> +β <sup>A</sup>	α <sup>A</sup> +β <sup>A</sup>	α <sup>A</sup> +β <sup>A</sup>	α <sup>A</sup> +β <sup>A</sup>	α <sup>A</sup> +β <sup>A</sup>	α <sup>A</sup> +β <sup>A</sup>
PCMB			α <sup>PMB</sup> + β <sup>PMB</sup> +A <sub>0</sub>		α <sup>PMB</sup> + β <sup>PMB</sup> +A <sub>0</sub>	
Hb A <sub>2</sub> (%)	2.1	2.8	2.5	2.4	2.9	2.2
Hb F (%)	0.9	n.d.	0.8	0.6	0.9	0.4
α/β syntetic ratio			1.40	0.95	1.05	

\* Extreme anisocytosis and poikilocytosis: basophilic stippling, erythroblasts

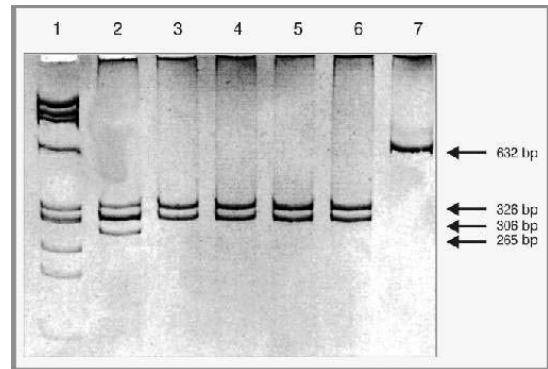
Peripheral blood smears showed extreme anisocytosis and poikilocytosis, hypochromia, basophilic stippling, and reticulocytosis. Large inclusion bodies were observed in peripheral blood upon staining with brilliant cresyl blue. The Hb A<sub>2</sub> and Hb F level were normal (2.7 % and 0.9 %, respectively). No abnormal Hb was detected by starch gel electrophoresis, starch gel electrophoresis in the presence of urea and polyacrylamide gel electrophoresis (PAGE) in the presence of urea-acetic acid-Triton X-100, and by different chromatographic methods. The heat and isoproponal stability tests showed no abnormal Hb. Treatment of haemolysate with PCMB did not show the presence of abnormal β chain. The α/β in vitro synthesis ratio was 1.40.

Therefore the molecular characterisation was carried out directly at the DNA level. When the sequence of exon 3 was compared with the sequence of a normal β globin gene, deletion of 6 bp of codons 137 (2<sup>nd</sup> and 3<sup>rd</sup> bp), 138 and 139 (the 1<sup>st</sup> bp) was found (- TGGCTA) (**Figure 1**).



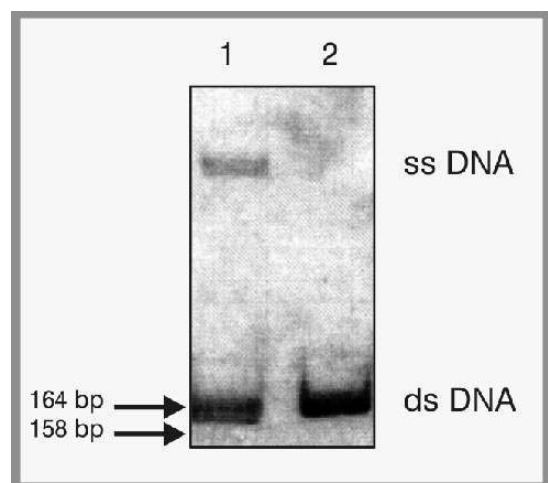
**Figure 1.** Electroferogram from the sequencing analysis of Exon 3 of the beta globin gene showing a deletion of 6 bp (-TGGCTA) at codons 137-139

This mutation leads to deletion of three amino acids (Val-Ala-Asn) and introduction of a new amino acid (Asp) at position 137, resulting in a chain 144 amino acid long. The sequence modification creates a new Hph I restriction site, and a 265 bp can be expected in addition to the normal 306 bp and 326 bp fragment. This was indeed observed for the proband while only 306 and 326 bp fragments were seen with the PCR product of the twin brother, mother and father (**Figure 2**).



**Figure 2.** Photograph of a PAGE of PCR amplified Exon 3 of the β-globin gene digested with Hph I showing a new restriction enzyme site created by 6 bp deletion at codons 137-139. Line 1: DNA marker IX (Boehringer); line 2: propositus; line 3: twin brother; line 4: mother; line 5: father, line 6: control; line 7: undigested PCR product

The RT/PCR product from the propositus separated on a non-denaturing 8 % polyacrylamide gel shows the presence of normal fragment of 164 bp and an abnormal fragment of 158 bp, which is 6 bp shorter than the normal PCR fragment (**Figure 3**). The relative amounts of β and β<sup>6nt</sup> transcripts were visually approximated and showed ratio β:β<sup>6nt</sup> of 60 % : 40 %.



**Figure 3.** Photograph of a PAGE of the RT / PCR products showing the presence of the normal and abnormal RNA transcripts. Line 1: propositus; line 2 : control

Analysis of restriction fragment length polymorphism at the β-globin gene cluster detected the haplotype I/IX. The patient has four α-globin genes.

Amino acid analysis of all the tryptic peptides of the  $\beta$  chain isolated by HPLC did not show the presence of abnormal Tp 14.

## DISCUSSION

This report describes a patient with thalassaemia major-like phenotype in whom DNA sequencing showed the presence of a new hyper-unstable  $\beta$ -chain variant caused by deletion of 6 bp at codons 137-139 and insertion of a new codon at position 137. This event eliminates three amino acids (Val-Ala-Asn) and introduces a new residue (Asp). Similar to the other previously described dominantly inherited  $\beta$ -thalassaemia (3-5) this new abnormal Hb manifest clinically in the heterozygous state with thalassaemias intermedia/major-like clinical picture. The Hb analyses data indicate that Hb Stara Zagora is synthesised but it undergoes a rapid turnover. The extremely rapid proteolysis of  $\beta^x$ -globin chain and the resulting excess of  $\alpha$ -chains most likely results in erythrocyte membrane damage which is responsible for the ineffective erythropoiesis expressed in transfusion dependent anaemia and iron overload. The high instability of this new abnormal Hb explains why protein analysis failed to detect it. The variant could not be detected by electrophoretic or chromatographic procedures; instability tests were normal and only Heinz bodies could be seen. The reason for the high instability of the molecule probably resides in the loss of Val-Ala-Asn. In fact, elimination of normal amino acid residues from codon 137 to 139 of the  $\beta$ -globin chain interferes with H-helix involved in  $\alpha 1\beta 1$  contact and  $\alpha 1\beta 2$  subunit interactions (20). The mutant globin chain could not interact with  $\alpha$ -globin chain to form a dimer and thus be removed by proteolysis. Similar hyper-unstable Hb (Hb Nijkerk) caused by deletion of 4 bp at codons 138-139 was described by van den Berg et al (21).

Of the 31 different dominantly inherited  $\beta$ -thalassaemia mutations, 17 are located in exon 3 of the  $\beta$ -globin gene (3-5). These mutations, which result in the production of elongated or truncated  $\beta$ -chains, manifest clinically in the heterozygous state and show a dominant transmission pattern. The clinical picture of our patient is not different from that of subjects with related mutations (reviewed in (22)).  $\beta^{\text{Stara Zagora}}$  is so unstable that it was not found as a synthesised polypeptide even at short times of incubation. The synthetic data are best explained by postulating that  $\alpha\beta$  dimer formation for  $\beta^{\text{Stara Zagora}}$  is completely

eliminated.

This new mutation, as is the case with the other described hyper-unstable haemoglobins, are rare alleles appearing in single or a small number of families from widely dispersed ethnic groups. The mutation creates an Hph I site which was used for identification. Both parents and his twin brother showed normal red cells indices, normal Hb F and Hb A<sub>2</sub> levels, which indicate that the abnormality is the result of a de novo event which has occurred in one of the gametes of the parents.

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