MOLECULAR BASIS OF $\beta$-THALASSAEMIA IN BULGARIA. AN UPDATE

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Abstract

$\beta$-thalassaemia is a relatively frequent inherited condition in the Mediterranean countries, especially on the Balkan Peninsula, and the incidence of $\beta$-thalassaemia among Bulgarians has been estimated to 2.5%.

Our purpose was to update the knowledge on the molecular basis of $\beta$-thalassaemia in Bulgaria and include some unpublished data.

The study was carried out at the University Children’s Clinic; University Eye Clinic; Trakia University – Stara Zagora, Bulgaria; Eye Hospital “Prof. Pashev”, Sofia, Bulgaria; Research Centre for Genetic Engineering and Biotechnology, Macedonian Academy of Science and Arts, Scopje, Republic of Macedonia; International Reference Laboratory for Haemoglobinopathies (IRLH) and for a period of 45 years 1752 patients were studied.

The inclusion criteria were clearly defined.

The most common form found is heterozygous $\beta$-thalassaemia. The ratio between the frequencies of $\beta^0$ and $\beta^+$-thalassaemia genes is 2:1, based both on the data for phenotypic expression and on molecular characterisation of thalassaemia genes. About half of all $\beta$-thalassaemia chromosomes have either the codon 39 (C→T) or the IVS-I-110 (G→A) mutations. Five additional mutations (IVS-I-1, cod 5, IVS-I-6, cod 6 and cod 8) occur at a frequency of 4 to 14%. During the period of investigations two new hyperunstable haemoglobins named Hb Stara Zagora and Hb Yambol were found.
β-thalassaemia is a severe disease among Bulgarians because the milder alleles, such as the three promoter mutations (-101,-87,5′ UTR +22), the IVS-I-6 mutation and the mutation in the polyadenylation site, which are known to be associated with thalassaemia intermedia, are observed for only 8.7% of the β-thalassaemic chromosomes. This is quite different from what has been observed in neighbouring countries, mainly because of the low frequency of IVS-I-6 (T→C) mutation among Bulgarian. The relatively high frequency of frame shifts at codons 5, 6, 8 and 8/9 was a surprise and may be specific for the population (of some provinces) of Bulgaria.

Key words: β-thalassaemia, molecular basis, gene mutation, homozygotes, compound heterozygotes

β-thalassaeamas are a group of inherited blood disorders caused by reduced or absent synthesis of the beta chains of haemoglobin resulting in variable phenotypes ranging from severe anemia to clinically asymptomatic individuals. It is a relatively frequent inherited condition in Bulgaria as in some Mediterranean countries and especially on the Balkan Peninsula. Since 1937, when the first patient with Cooley’s anemia was described [1], many reports with new cases of the disease have followed [2–5]. Gradually, β-thalassaemia was outlined as a significant medical as well as social problem that attracts the attention of medical community. Over the past 45 years, population studies have been carried out in most parts of the country [6, 7].

The investigation of β-thalassaemia in Bulgaria can conditionally be divided in two main periods. The first period was from 1937, when the first cases with Cooley’s anemia were described, up to 1965. This is a period of clinical investigation mainly of the diversity of β-thalassaemia, observed in different regions of Bulgaria.

The second period began in 1965 and continues nowadays. It is characterized with genetically-biochemical investigation of β-thalassaemia, with understanding the molecular basis of β-thalassaemia syndromes and resembling conditions in Bulgaria. This period of investigation is closely related with the names of Prof. Huisman (Department of Cell and Molecular Biology; Medical College of Georgia, Augusta, USA) and Academician G. D. Efremov (Research Centre for Genetic Engineering and Biotechnology, Macedonian Academy of Science and Arts, Scopje, Republic of Macedonija, International Reference Laboratory for Haemoglobinopathies (IRLH)).

After the description of a number of clinical cases with β-thalassaemia during the first period, some population studies were carried out on the incidence of thalassaemia in Bulgaria. Using different methods of investigation, a significant variability of carrying the gene was found (from 0.05% to 19.9%) [4–9].

During the second period of investigation, using modern and precise methods, the average frequency of monogenic carrying was found to be about 2.5% [5, 6]. There are some differences between the various regions of the country.
The carrying of thalassaemia gene is relatively higher in South–West, South, and South–East Bulgaria, and in the region of Black Sea as well.

Our first results concerning the molecular basis of β-thalassaemia in Bulgaria were published in 1990 [10]. Here we update our results on the molecular basis of β-thalassaemia in Bulgaria and include some unpublished data.

Materials and methods. During the past 45 years, we studied 1752 patients with signs of hemolysis and/or low red blood cell (RBC) indices for the presence of thalassaeomias and other haemoglobinopathies. Among them 743 (42.4%) had thalassaemia or related condition, of which 155 (8.8%) were thalassaemia major patients, 9 (0.5%) – thalassaemia intermedia, 205 (11.7%) – heterozygous relatives and 373 (21.3%) – unrelated β-thalassaemia heterozygotes. Most patients who attended the Pediatric Clinic at the University of Stara Zagora and some other clinics were seen in local clinics of south-central provinces of Bulgaria and the region of the Black Sea. Nearly all patients were children or people in early adulthood. In addition, we studied blood samples from 875 newborn babies. Informed consent was obtained.

Methods for detection and characterization of thalassemias and abnormal haemoglobins were those universally accepted [11, 12] and consisted of: determination of RBC indices and morphology; osmotic fragility test [13]; quantitation of Hb A₂ by microcolumn chromatography [14]; quantitation of Hb F by an alkali denaturation procedure [11]; starch gel electrophoresis [14] and HPLC. Whenever indicated, specific studies such as tests for unstable Hbs [14], isoelectrofocusing, structural characterization of the variant Hbs, and in vitro biosynthetic studies were performed [11, 14]. Gene mapping analysis, dot-blot hybridization of polymerase chain reaction (PCR) amplified DNA with specific probes, and sequencing of the amplified DNA followed the methodology described before [16, 17]. We used the routine techniques for determining eye status, cardio-vascular and pulmonary systems [18] in patients with thalassaemia major and other clinical presentations caused by different genotypes.

Results and discussion. Frequencies. Figure 1 lists and shows the 21 different forms of thalassaeomias and related conditions detected, characterized and registered in our institutions and the frequencies of 18 different β-thalassaemia alleles studied.

The most common form is a heterozygous β-thalassaemia that has been observed in 546 patients, of which 374 β⁺⁻thalassaemia, 163 β⁺⁺thalassaemia and 9 βsilent-thalassaemia. The ratio between the frequencies of β⁺⁻ and β⁺⁺-thalassaemia genes is 2:1, based both on the data for phenotypic expression and on molecular characterization of the thalassaemia genes.

Twenty-five per cent of the β-thalassaemia major patients carried the nonsense codon 39 (C→T) mutation, and 23.7% the IVS I-110 (G→A) mutation (29.1% and 23.0% respectively in the survey of the heterozygotes). The third most frequently occurring variant is IVS I-1 (G→A) mutation (14.2%), and then

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codon 5 (8.4%) and IVS I-6 (6.7%). Thus these five mutations account for about 80% of the β-thalassaemia genes, and along with codon 6, codon 8 and codon 8/9 mutation, these eight mutations account for more than 90% of the mutations observed in Bulgaria. Promoter mutations were relatively rare; only two chromosomes with C→T at −101, nine chromosomes with C→G at −87 and two chromosomes with T→A at −30 were observed. Thus the occurrence of alleles with relatively mild clinical consequences (i.e. C→T at −101; C→G at −87; T→A at −30; 5’UTR; +22 (G→A), T→C at IVS 1–6, and A→G at the polyadenilation signal (poly site) was 8.5%.

**β-thalassaemia homozygotes.** Among the 131 thalassaemia major and intermediate patients, 44 different genotypes were found. Only 32 were found to be homozygotes – this reflects the large number of alleles which occurred at the relatively high incidence. Twelve patients were homozygous for codon 39 mutation, six – each for codon 8 and IVS I-110 mutations, two – each for
codon 5, IVS I-1 and IVS II-745 mutations, and one – for codon 8/9 and IVS I-6 mutation. The severity of the clinical presentation depends on the genetic defect. The genotypes causing the severe form of the disease presented higher diversity of ocular symptoms, mainly the ones causing chronic hypoxia of the retina.

**Compound heterozygotes.** One hundred and fourteen patients were double heterozygotes for β-thalassaemia. Compound heterozygosity for codon 39 and IVS 1-110 was found in 19 patients, and compound heterozygosity for IVS 1-110 and IVS 1-6, and IVS 1-110 and codon 5 were found in six and five patients respectively. Various other combinations were observed in one to four patients, many causing severe disease and a blood transfusion dependency. Only a few patients had a somewhat less severe disease, notably those with – 101/codon 39 combination (one patient), – 87/codon 5 and – 87/ codon 39 (one patient each), – 87/codon 6 (two patients), –30/IVS 1–6 (one patient), and PolyA/IVS1-110 combination (one patient).

**Combinations of β-thalassaemia with an abnormal Hb.** Seven patients with Hb Lepore Boston Washington/ (δ 87 GLUT δ VS II−8)β-thalassaemia and eight patients with Hb O Arab/β-thalassaemia were studied. The β-thalassaemia mutations were: codon 39 (five), IVS I-1 (two), IVS I-6 (two), IVS I-110 (four), codon 8 (one) and IVS II-745 (one). The clinical severity of the disease varied depending on the β-thalassaemia allele.

The frequency of carrying the gene for Hb 0 Arab is highest in the corner of the south–eastern area of the region.

We found single cases in other regions of the countries, but when their descent was traced it was found to be from the region of Burgas.

About the incidence of Hb 0 Arab based on the population migration through the history of the Balkan countries, Efremov thought that the gene might be introduced in the region in ancient time or during the Ottoman Empire period.

**β-thalassaemia heterozygotes.** A total of 546 subjects were heterozygotes for β-thalassaemia, of which 374 (51.7%) β0-thalassaemia, 163 (22.5%) β+thalassaemia and 9 subjects (1.2%) βsilent-thalassaemia. The haematological and haemoglobin composition data for the β0- and β+thalassaemia are given in Fig. 2A and 2B.

As it can be expected, the haemoglobin concentration and the RBC indices are slightly lower in β0-thalassaemia than in β+thalassaemia.

**δβ-thalassaemia.** This condition is relatively rare in Bulgaria. It has been observed in homozygous state in one patient, in combination with β-thalassaemia in six patients and in heterozygous state in three cases.

Gene mapping analysis in most of the cases with this condition showed that the δβ-thalassaemia among Bulgarians is of Sicilian type.

**Dominant β-thalassaemia.** Very recently we have described two new dominant β-thalassaemia mutations [26–28]. The first one is caused by six-based pair (bp), deletion (-TGGGT A) at codons 137 (the second and third bp, 138 and 139...
Fig. 2. A) Haematological and haemoglobin composition data of Bulgarian heterozygotes for β-thalassaemia in males; B) Haematological and haemoglobin composition data of Bulgarian heterozygotes for β-thalassaemia in females.

(the first bp), forming a new codon at position 137 (GAT). This event eliminates three amino acids (Val-Ala-Asn) and introduces a new residue (Asp) (Fig. 3).

This hyper-unstable haemoglobin named Stara Zagora was found in a two-year-old boy, as a de novo mutation. He is twin but his brother is haematologically healthy.

The other dominant β-thalassaemia was caused by a new hyper-unstable haemoglobin named Hb Yambol, discovered in a girl who developed hemolytic anemia at three months of age.
Fig. 3. Electroforegram from the sequencing analysis of Exon 3 of the beta globin gene showing a deletion of 6 bp (-TGGCTA) at codon 137–139

Fig. 4. Sequence analysis of the $\beta$-Yambol globin gene. a) cDNA sequence showing a deletion of the first 10 nts of exon 3 and an insertion of 25 nts of the same location; b) Part of $\beta$-Yambol IVS-II with an insertion of 23 nts; c) Part of $\beta$-Yambol IVS-II and exon 3, showing a deletion of 310 nts (300 nts of IVS-II and 10 nts of exon 3) and an insertion of 28 nts at the junction of the deletion

The mutation is a deletion of 285 bp (from IVS II-575 to the 2nd nt of codon 108), triplication of 12 nt sequence of IVS II (from nt 536) and insertion of 28 nt with reverse homology from the 3' UTR of the β-globin gene (from nt 3695 to nt 3724, 3' to the termination codon). At the protein level, this mutation leads to a deletion of 4 amino-acid residues (Leu-Leu-Gly-Asn) and an insertion of 9 amino-acid residues (Val-Pro-Ser-Val-Thr-Leu-Phe-Phe-Asp), creating an abnormal elongated β-chain of 151 amino acid residues (Figs 4, 5).

The sites of the insertion/deletion are clearly indicated. The different pairs of short direct (bold) and indirect repeats (bold and shaded) thought to have mediated strand mispairing are highlighted. Wild type genomic sequences (β-globine gene and 3' flanking sequences) were extracted from Gene Bank NT009237.

α-thalassaemia. The frequency of α-thalassaemia in Bulgaria is relatively low. Hb H disease has not been observed. Testing of 875 cord blood samples shows high levels of Hb Bart’s (α-thalassaemia1) in 0.5%, and low levels of α-thalassaemia2 in 1.6% of the screened babies.

Conclusion. The incidence of β-thalassaemia among Bulgarians has been estimated at 2.5% [8]. The distribution pattern has not been clearly defined, but the highest incidence occurs among Bulgarians in the south, where the country borders with Greece and European Turkey. That is actually where the patients and their parents who participated in this 45-year-follow up study came from. The frequency data listed in Figure and Table 2 indicate that about half of all β-thalassaemia chromosomes have either the codon 39 (C→T) or the IVS 1-110 (G→A) mutations. Five additional mutations (IVS I-1, codon 5, IVS I-6, codon 6 and codon 8) occur at a frequency of 4 to 14%. This rather broad spectrum is not favourable for any institution involved in prenatal diagnostic programmes because testing for multiple alleles is required.
β-thalassaemia is a severe disease among Bulgarians because the milder alleles, such as the three promoter mutations (-101, -87, -30), 5’ UTR + 22, the IVS I-6 mutation and the mutation in the polyadenylation site, which are known to be associated with thalassaemias intermedia, were observed for only 8.7% of the β-thalassaemic chromosomes. This is quite different from what has been observed in neighbouring countries, mainly because of the low frequency of the IVS I-6 (T→C) mutation among Bulgarians. The relatively high frequencies of frameshifts at codons 5, 6, 8 and 8/9 (together accounting for 19.5% of all β-thalassaemia alleles) were a surprise and may be specific for the population (of some provinces) of Bulgaria.

REFERENCES


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