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BIOCHEMICAL PHENOTYPE AND ORIGIN OF THE THREE MOST COMMON BETA-THALASSEMIA MUTATIONS IN SERBIA

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Summary: Molecular (DNA) characterization of thalassemia is the most reliable methodology for the diagnosis of this group of diseases. As thalassemias are very heterogeneous, hematological data and additional biochemical analysis are essential for their differential diagnosis. In this paper we present hematological and biochemical characteristics of the carriers of three most common beta-thalassemia mutations in Serbia (Hb Lepore, $\beta^{\circ}39$ and $\beta^+IVS-I-110$), to be taken into consideration as the initial step of the diagnostic approach to the thalassemia patients. Also, this paper represents a detailed survey of the diversity of β -globin gene haplotypes in carriers of the most common β -thalassemia mutations and normal betaA/betaA individuals of Serbian descent. A novel haplotype associated with Hb Lepore-Boston-Washington gene has been identified in Serbian population. These data support the hypothesis of multicentric origin of this mutation. The mutation has arisen *de novo* in the chromosomal background characteristic for Serbian population. Additionally, we have shown that two most common Mediterranean mutations, $\beta^{\circ}39$ and $\beta^+IVS-I-110$, have probably been introduced into Serbian population from Italy and Turkey, respectively, through historically documented migrations and settlements.

Key words: β -thalassemia, haplotype, Hb Lepore

Introduction

The β -globin gene cluster is located on chromosome 11. The alignment of genes in human β -globin locus represents the order of gene expression during ontogenic development: embryonal genes (ϵ) are located at the 5' end, then fetal genes ($A\gamma$, $G\gamma$) and finally, at the 3' end, adult genes (δ , β). Thalassemia syndromes are a group of hereditary disorders generally caused by mutations in globin genes. In Serbia, thalassemia syndromes are less frequent than in adjacent Mediterranean countries. The overall frequency of thalassemia syndromes in Serbia is 1.9% (1).

Since 1998 thalassemia syndromes have been systematically characterized on molecular level in the population of Serbia (2). Nine different mutations have been detected: seven β -thalassemic mutations and two thalassemic hemoglobin (Hb) variants (Hb Lepore, Hb Sabine), among which 70% were Hb Lepore, $\beta^{\circ}39$ and $\beta^+IVS-I-110$ (2). Two studies showed that Hb Lepore is the most common cause of thalassemia in Serbia (30%) (1, 2).

Hb Lepore is an abnormal thalassemic hemoglobin variant, which consists of normal α -globin chains and fused $\delta\beta$ -globin chains. The $\delta\beta$ -globin chain is a product of an unequal recombination that joins the proximal end of the δ -globin gene with the distal end of the β -globin gene (3). Three types of Hb Lepore, that differ in the position at which the transition from δ to β DNA occurs, have been described: Hb Lepore-Hollandia, Hb Lepore-Baltimore and Hb Lepore-Boston-Washington (Hb Lepore BW) (3).

Substitution (C \rightarrow T) alters codon 39 to a stop codon. Such chain termination (nonsense) mutation

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aborts mRNA translation and leads to synthesis of a truncated polypeptide (4). Therefore, codon 39 results in β^0 thalassemic phenotype. Substitution (G→A) at position 110 in intron I (IVS-I-110) creates an alternative splice site. The incorrectly spliced mRNA leads to premature termination of translation but allows accumulation of some amount of normal globin mRNA (β^+ thalassemic phenotype) (5).

In this study we have analyzed hematological data and Hb content (Hb A, Hb A₂ and Hb F) in carriers of three most common beta-thalassemia mutations in Serbia. Also, we present a detailed study of the diversity of β -globin gene haplotypes in normal betaA/betaA and thalassemic individuals of Serbian descent, conducted with the aim of defining the origin of beta-thalassemia in Serbia.

The β -globin gene cluster is highly polymorphic. The pattern or combination of polymorphic restriction sites in β -globin locus for any chromosome is called a haplotype (6). There are nine common haplotypes (I-IX) known to be present in different Mediterranean populations with significant percentage (6). Additionally, four beta globin intragenic single base polymorphisms have led to the definition of three different beta globin gene frameworks (6). Haplotypes and frameworks of β -globin gene cluster are widely used nowadays to determine the chromosomal background on which a mutation arose, as well as its geographical pattern.

Material and Methods

Forty-five Serbian patients from 20 unrelated families affected with β -thalassemia, were studied: 21 Hb Lepore BW β -thalassemic variant carriers (from 8 unrelated families), 14 β^0 39 β -thalassemia mutation carriers (from 7 unrelated families) and 10 β^+ IVS-I-110 mutation carriers (from 5 unrelated families). Healthy subjects related to the carriers were also analyzed.

Hematological parameters were obtained using an automated cell counter. Abnormal hemoglobin was detected by electrophoresis on cellulose acetate in Tris-borate-EDTA (pH 8.4) (7). Hb F was quantified by standard methods (8, 9) and Hb A₂ and Hb Lepore were estimated by elution from celogel electrophoretic strips.

DNA was extracted from peripheral blood collected with sodium citrate or EDTA as an anticoagulant (10). DNA samples were used as templates in polymerase chain reaction (PCR). For each sample eight PCR-RFLPs were performed, one for each polymorphism (*HincII/ε*, *XmnI/5'Gγ*, *HindIII/Gγ*, *HindIII/Aγ*, *HincII/ψβ*, *HincII/3'ψβ*, *Avall/β*, *BamHI/3'β*). The conditions for PCR amplification were as described previously (11, 12), with changes in temperature of annealing for ϵ , 5'Gγ, Gγ, Aγ, ψβ, 3'ψβ, β, 3'β DNA

fragments containing β -globin gene cluster polymorphic sites: 55, 55, 60, 55, 61, 61, 57 and 53 °C respectively. For haplotype analysis the polymorphic restriction enzyme sites were determined by digestion of PCR amplified fragments using the methodology of Sutton et al (12). Polymorphic sites were detected by agarose gel electrophoresis. The framework base substitutions were identified by dideoxy chain termination method using fluorescently labeled dideoxy nucleotides (Pharmacia Biotech, Uppsala, Sweden) on an automated DNA sequencer (ALFexpress DNA Sequencer, Pharmacia Biotech) using the primer 5'-AA TCA TTC GTC TGT TTC CCA-3'.

Results

All carriers of Hb Lepore had a clinical phenotype of thalassemia trait. Their MCV and MCH values were reduced (Table I). Hb A₂ level was normal or decreased, while Hb F was slightly increased. Hb analysis by electrophoresis on cellulose acetate revealed the presence of an abnormal Hb fraction, later characterized as Hb Lepore by PCR analysis. Heterozygous carriers of β^0 39 mutation had evident anemia, but unusually mild microcytosis and hypochromia for this type of mutation (β^0). As expected, Hb A₂ and Hb F were markedly elevated (5% and 3.2%, respectively). All carriers of β^+ IVS-I-110 mutation were clinically asymptomatic. They presented with relatively mild anemia and moderately reduced MCV and MCH values. Both Hb A₂ and Hb F levels were slightly elevated.

In order to elucidate the origin of the most common thalassemic mutations in the population of Serbia, haplotype analysis of Hb Lepore BW, β^0 39 and β^+ IVS-I-110 mutation carriers was performed. We have studied eight linked polymorphic restriction sites along β -globin gene cluster (Figure 1). In order to establish the haplotype of thalassemic chromosomes with certainty, nonaffected relatives were included in the study.

Four different haplotypes associated with β -thalassemia mutations were detected: two of them were typical of Mediterranean countries (I and V), and two autochthonous haplotypes, specific for Serbian population, were discovered as well (haplotypes A and B) (Table II).

Table I Hematological data (average values and standard deviations), Hb A₂ and Hb F values for β -thalassemia heterozygotes

Mutation	Gender	No.	Hb (g/L)	MCV (fL)	MCH (pg)	Hb A ₂ (%)	HbF (%)
Hb Lepore (β fusion)	M	11	114.5 ± 9.02	66.2 ± 3.51	20.35 ± 1.50	1.95 ± 0.82	2.15 ± 0.60
	F	10	108.5 ± 5.22	66.0 ± 1.53	20.55 ± 1.75	1.84 ± 0.62	2.16 ± 0.51
Codon 39 (C→T)	M	7	109.0 ± 1.50	67.4 ± 3.90	22.6 ± 0.98	5.0 ± 0.25	4.20 ± 4.08
	F	7	91.0 ± 0.73	67.0 ± 0.33	22.1 ± 0.33	4.6 ± 0.50	3.87 ± 0.43
IVS I-110 (G→A)	M	5	128.5 ± 0.50	66.1 ± 1.66	20.8 ± 0.50	4.3 ± 0.15	2.33 ± 0.77
	F	5	126.0 ± 0.90	64.1 ± 3.35	20.9 ± 1.05	4.2 ± 0.20	2.30 ± 0.60

Table II Haplotypes detected in the healthy and thalassemic population of Serbia

	5' subhaplotype						3' subhaplotype	
	ϵ	5'G γ	G γ	A γ	$\psi\beta_1$	3' $\psi\beta$	β	3' β
Haplotype I [4]	+	-	-	-	-	-	+	+
Haplotype V [4]	+	-	-	-	-	-	+	-
Haplotype A	+	-	-	+	-	-	+	-
Haplotype B	+	-	-	+	-	-	+	+

Polymorphic restriction sites: *HincII*/ ϵ , *XmnI*/5'G γ , *HindIII*/G γ , *HindIII*/A γ , *HincII*/ $\psi\beta_1$, *HincII*/3' $\psi\beta$, *AvaI*/ β , *BamHI*/3' β

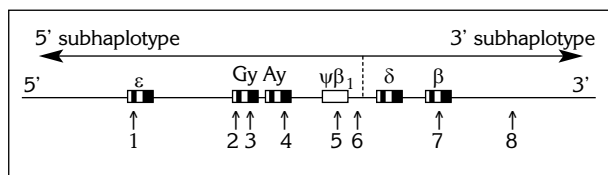


Figure 1. The location of eight polymorphic restriction sites in the β -globin gene cluster. Sites are numbered from 5' to 3': 1-*HincII*/ ϵ , 2-*XmnI*/5'G γ , 3-*HindIII*/G γ , 4-*HindIII*/A γ , 5-*HincII*/ $\psi\beta_1$, 6-*HincII*/3' $\psi\beta$, 7-*AvaI*/ β , 8-*BamHI*/3' β ; 5' and 3' subhaplotypes are defined by restriction sites 1-6 and 7, 8, respectively.

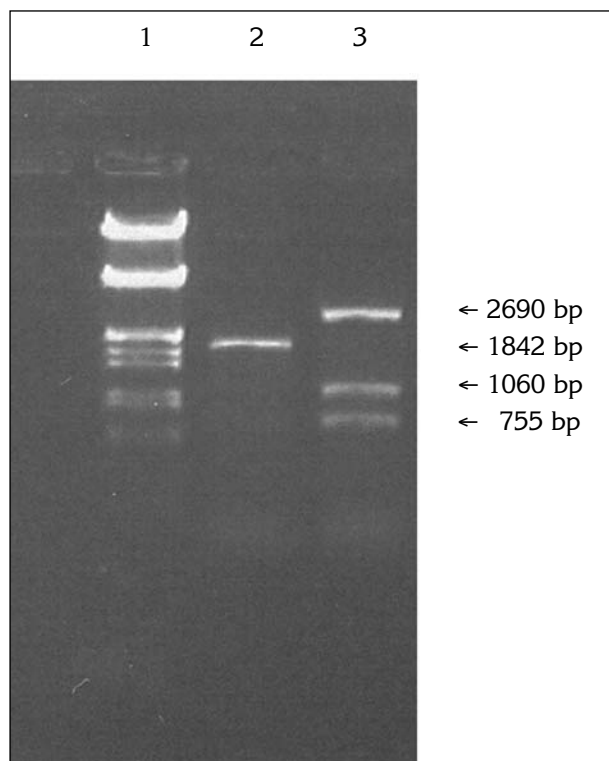


Figure 2. Detection of *HindIII*/A γ polymorphic site by 1% agarose gel electrophoresis. 1. λ /*HindIII*/*EcoRI* DNA marker; 2. undigested PCR fragment; 3. homozygote for *HindIII*/A γ polymorphism; 2690bp indicates pUC19 plasmid DNA digested with *HindIII* (control of the restriction enzyme activity)

All analyzed Lepore chromosomes were of the identical haplotype: haplotype A. Comparing this haplotype with Hb Lepore BW haplotypes reported to date showed that it differs from all of them. Thus, in this study a novel haplotype associated with Hb Lepore BW mutation was identified. The specificity of this haplotype is the presence of *HindIII*/A γ restriction site (Figure 2).

Haplotype analysis revealed that β^0 39 thalassemia mutation is mostly associated with haplotype I and framework 1. However, in one family, β^0 39 chromosomes were found to be associated with framework 2. Two different haplotypes were associated with β^+ IVS-I-110 thalassemic mutation: haplotype I and haplotype B. Also, we have detected the presence of haplotypes I and V, as well as haplotypes A and B specific for Serbian population, in normal betaA/betaA individuals.

Discussion

The results presented in this paper may help the clinicians as a guideline for differential diagnostics of thalassemia trait. The hematological values of particular importance are: MCV lower than 70fL and MCH lower than 24pg. If these values are consistently decreased (despite Fe supplementation therapy) in anemic patient, hemoglobin electrophoresis is recommended, followed by DNA analysis.

Haplotype analyses we present in this paper have been carried out on β -thalassemia carriers of common Mediterranean mutations (Hb Lepore BW, β^0 39, β^+ IVS-I-110). A number of similar studies have already provided insights into origin and distribution of these mutations. Beta-globin gene haplotypes associated with thalassemic mutations were sporadically studied in former Yugoslavia, too (13). However, chromosomal background of β -thalassemia mutations in Serbian population demonstrates specificity, not previously reported.

In this paper we present a novel haplotype associated with Hb Lepore BW gene. Hb Lepore BW is common in Mediterranean countries. Up to date, 6 haplotypes associated with Hb Lepore BW chromosomes were identified: haplotype V in 34 families of Spanish, Yugoslavian and Italian origin (14-16), haplotype I in 23 Italian families (15), haplotypes VI and VII in Italian families (15) and two unique haplotypes (in a Hungarian individual and in one Black family) (15). A multicentric origin of this mutation has been suggested (16). In order to elucidate the origin of Hb Lepore BW mutation in Serbia, the 5' and 3' subhaplotypes were taken into consideration as independent entities (see Figure 1). We have detected two different 5' subhaplotypes: the first one, typical for both I and V haplotypes, and the second, autochthonous 5' subhaplotype present in both haplotypes A and B. There were also two 3' subhaplotypes: the one typical

for haplotype I and the other typical for haplotype V (see *Table II*).

All Serbian Hb Lepore BW genes are associated with autochthonous 5' subhaplotype and 3' subhaplotype typical for haplotype V. We have previously shown that they are also associated with framework 2 (17). The Hb Lepore BW gene associated with haplotype V and framework 2 was virtually the only type found in Eastern Italy and former Yugoslavia (13, 16). Considering the fact that two different haplotypes containing autochthonous 5' subhaplotype (A and B) as well as haplotype V were detected in healthy Serbian population, two possible hypotheses could explain the origin of Hb Lepore BW mutation on a novel chromosomal background.

There is a possibility that unequal recombination or gene conversion occurred between two chromosomes with haplotypes A or B and Hb Lepore mutation associated with haplotype V, respectively, producing new chromosomal background for Hb Lepore mutation. The fact that the probable site of recombination could have been in the 9,1 kb segment, which has been defined by Chakravarti *et al.* (18) as a hot spot region for nonuniform recombination in β -globin gene cluster, supports this hypothesis.

However, the mutation might have arisen *de novo*, by a single independent mutational event in a healthy individual with haplotype A and spread in the population. The high frequency of Hb Lepore BW hemoglobin variant in Serbian population, the homogeneity of Hb Lepore BW haplotype (no haplotype V was detected), as well as its uniqueness, suggest its independent origin. The finding that Hb Lepore BW chromosomes are concentrated in particular geographical region of Serbia also supports this hypothesis.

It has been proposed that $\beta^{\circ}39$ mutation originates from Italy (19). Our data suggest Western Me-

diterranean (Italian) origin of this mutation in Serbia. Chromosomal background of $\beta^{\circ}39$ mutation in our population is similar to the one found in other Mediterranean countries. However, in one family $\beta^{\circ}39$ chromosomes are found to be associated with framework 2. Although mutation spread almost never involves a change of β -globin gene framework, the exception has been detected (19). To the best of our knowledge, this is the first report of the association between framework 2 and $\beta^{\circ}39$ mutation. This example of mutation spread from one beta-globin gene framework to another, within a population, may be due to inter-allelic gene conversion events or recurrent mutation (19).

β^+ IVS-I-110 mutation is previously reported to be of Eastern Mediterranean (Turkish) origin and associated with haplotype I (20). In this study, β^+ IVS-I-110 mutation is found to be associated with two different haplotypes. Actually, these two haplotypes differ only in their 5' subhaplotypes: one is typical for haplotype I, and the other is 5' subhaplotype from haplotypes A or B. The likelihood that this haplotype dimorphism associated with the same β -thalassemia mutation is a consequence of random mutation is very low. The diversity of haplotypes associated with β^+ IVS-I-110 mutation, would then be generated by recombination events (crossing-over or gene conversion) between the original β -thalassemic chromosome (haplotype I) and the 5' subhaplotype characteristic for normal population of Serbia.

Although thalassemia syndromes are sporadic disorders in Serbia, they reflect numerous historically documented migrations over Balkan Peninsula and yet show autochthonous features.

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BIOHEMIJSKI FENOTIP I POREKLO TRI NAJČEŠĆE BETA-TALASEMIJSKE MUTACIJE U SRBIJI

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Kratak sadržaj: Molekularna (DNK) karakterizacija talasemija je najpouzdaniji metod za dijagnostiku ove grupe oboljenja. Kako su talasemije vrlo heterogene, hematološki podaci i dodatne biohemijske analize su od esencijalne važnosti za njihovu diferencijalnu dijagnostiku. U ovom radu su prikazani hematološki parametri i biohemijske karakteristike nosilaca tri najčešće β -talasemijske mutacije u Srbiji (Hb Lepore, $\beta^{\circ}39$ i $\beta^{+IVS-I-110}$) koje je neophodno uzeti u obzir u početnom koraku postavljanja dijagnoze kod talasemičnih pacijenata. Pored toga, ovaj rad predstavlja detaljnu analizu diverziteta haplotipova β -globinskog lokusa kod nosilaca najčešćih β -talasemijskih mutacija i zdravih betaA/betaA osoba u Srbiji. Identifikovan je novi haplotip asociiran sa Hb Lepore-Boston-Vašington genom u srpskoj populaciji. Ovi podaci idu u prilog hipotezi o multicentričnom poreklu ove mutacije. Mutacija je nastala *de novo* u hromozomskom kontekstu koji je karakterističan za populaciju u Srbiji. Takođe je pokazano da su dve najčešće mediteranske mutacije, $\beta^{\circ}39$ i $\beta^{+IVS-I-110}$, verovatno »uvezene« u populaciju Srbije iz Italije, odnosno Turske, istorijskim migracijama i seobama naroda.

Ključne reči: β -talasemija, haplotip, Hb Lepore

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