The Relevance of β-Thalassemia Heterozygosity in Pediatric Clinical Practice: Croatian Experience

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Article

The Relevance of β-Thalassemia Heterozygosity in Pediatric Clinical Practice: Croatian Experience

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Abstract: Thalassemia syndromes are common monogenic disorders that represent a significant global health issue. No systematic epidemiological and molecular investigations on thalassemias in Croatian population have been reported to date. This prospective study included 70 children with a presumptive diagnosis of thalassemia, and their 42 first-degree relatives. Molecular characterization was performed using direct sequencing and gap-PCR methods. We identified 46 (30 children and 16 first-degree relatives) β-thalassemia heterozygous carriers from 24 unrelated families, carrying eight different mutations and one hemoglobin variant. Five variants account for approximately 85% of all affected β-globin alleles: Hb Lepore-Boston-Washington (32,6%), HBB:c.93-21G>A (19,6%), HBB:c.315+1G>A (13,1%), HBB:c.92+1G>A (10,9%), and HBB:c.92+6T>C variant (8,7%). A need for more detailed genetic profiling of β-thalassemia carriers is emphasized since genetic modifiers can significantly impact their phenotype. Our study provides important new insights into the relevance of β-thalassemia heterozygosity in the pediatric clinical practice.

Keywords: beta-thalassemia; genotype; screening; Croatia; pediatric

1. Introduction

Beta-thalassemias are a heterogenous group of inherited blood disorders that result from defective synthesis of β-globin chains leading to ineffective erythropoiesis and hemolysis. Beta-thalassemia includes three main forms: thalassemia major (also called Cooley anemia or Mediterranean anemia), thalassemia intermedia and thalassemia minor (also known as heterozygous β-thalassemia, and β-thalassemia trait) [1] (pp. 609-619). The severity of the disease is determined mainly by the extent to which synthesis of β-globin chains of hemoglobin is reduced (β+ and β++) or absent (β0), resulting from the underlying molecular defects on β-globin (HBB) gene located on chromosome 11. The clinical spectrum is wide, ranging from asymptomatic individuals with mild microcytic hypochromic anemia to severe transfusion-dependent thalassemia. The remarkable phenotypic variability is primary due to striking variations in β-globin (HBB) gene, with over 300 variants reported [2].

Beta-thalassemia is one of the most common monogenic disorders worldwide, with approximately 1.5% of the global population (80-90 million people) being carriers and 60,000 symptomatic newborns annually [1,3,4]. It is more prevalent in tropical and subtropical areas, extending from sub-Saharan Africa, the Mediterranean, the Middle East, and Southeast Asia ("thalassemia belt") due to the presence of malaria [5].

Croatia is located at the crossroads of Central Europe, the Balkans, and the Mediterranean [6]. Although at the edge of the traditional thalassemia belt, neither systemic epidemiological nor genetic studies of thalassemia syndromes in the Croatian population have been performed, and there are

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only a few case reports and one study with a very limited number of patients [7]. This study was performed to analyze the spectrum of the β-globin gene variants and to provide baseline data useful in launching carrier screening, genetic counseling, and prenatal diagnosis of β-thalassemias in Croatia. Besides, we highlight the importance of molecular diagnosis of heterozygous β-thalassemia and its implication on patients' health.

2. Materials and Methods

2.1. Subjects

One hundred and twelve patients (47 females and 65 males) from 65 unrelated families were included in the study. From July 1, 2021, to June 30, 2023, 70 children (mean age 9,9 years; range 0,4 - 17,1 years) with microcytic hypochromic anemia and/or elevated levels of hemoglobin (Hb) A2 with a presumptive diagnosis of thalassemia were recruited from the Clinical Hospital Centre (CHC) Rijeka, Croatia, and their 42 first-degree relatives with the history of anemia. DNA analysis was performed at the Institute of Molecular Genetics and Genetic Engineering, Belgrade, Serbia. The study was approved by the Ethical Committee of CHC Rijeka (No 2170-29-02/1-21-2; May 31, 2021).

2.2. Methods

Hematological parameters were obtained by an automated hematology analyzer (Sysmex XN-1000; Sysmex Europe GmbH, Norderstedt, Germany).

Hb fractions were detected by capillary zone electrophoresis method using the automated Sebia Capillarys 2 Flex Piercing System (Sebia, Lisses, France). EDTA-whole blood specimens were subjected to onboard hemolysis prior to capillary injection and separated by electrophoretic mobility in an alkaline buffer. Quantitation of eluted fractions was performed spectrophotometrically at 415 nm, and peaks (electrophoregrams) were evaluated visually based on their migration within defined zones.

Genomic DNA was obtained from peripheral blood (3 ml) collected in the sodium citrate tubes. The polymerase chain reaction (PCR) primers and conditions used to amplify the *HBB* gene are available upon request. The PCR products were purified with a QIAquick PCR purification kit (Qiagen Inc.) and used for direct PCR product sequencing in both directions with a BigDye terminator kit (Applied Biosystems) using the SeqStudio Genetic Analyzer (Thermo Fisher Scientific) according to the manufacturer instructions with the same primers used for PCR amplification. Detection of the Hb variant Lepore-Boston-Washington (Hb Lepore-BW) was performed by gap-PCR analysis as previously described [8].

2.3. Statistical Analysis

Categorical variables are presented as absolute numbers and frequencies. Continuous variables, presented as medians or means with SEM (standard error of the mean), were analyzed by Student's t-test. Shapiro-Wilk test was used to assess the data distribution.

Statistical analysis was performed using SPSS 21.0 software (IBM). For all analyses, p-values were 2-tailed, and the significance was defined as $p<0.05$.

3. Results

3.1. Genotype Analysis

Out of 112 cases belonging to 65 unrelated families, we identified eight different thalassemia mutations and one Hb variant in total of 46 cases (30 children [mean age 8,9 years; range 0,4 - 17,1 years] and 16 first-degree relatives; 21 females and 25 males) from 24 unrelated families (Table 1). All variants were detected in the heterozygous form.

Hb Lepore-BW was the most common cause of thalassemia in our cohort, with the frequency of 32,6%. More than 19% of patients carried HBB:c.93-21G>A variant and 13,1% carried HBB:c.315+1G>A variant. The third most frequent variant was HBB:c.92+1G>A (10,9%), followed by HBB:c.92+6T>C variant (8,7%). Altogether, these five variants accounted for up to 85% of all affected β-globin alleles. Two rare β-globin gene variants were also detected, Hb Monroe with the frequency of 4,3% and polyA (A>G) with the frequency of 2,2%, as presented in **Table 1**.

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Table 1. Frequencies of the *HBB* variants causing β-thalassemia syndromes in Croatia.

Variant	HGVS* nomenclature	Type of mutation	Families		Chromosomes	
			n	%	n	%
Hb Lepore-BW	NG_000007.3:g.63632_71046del	Hb variant	7	29,2	15	32,6
IVSI-110	HBB:c.93-21G>A	β^+	5	20,8	9	19,6
IVSII-1	HBB:c.315+1G>A	β^0	4	16,6	6	13,1
IVSI-1	HBB:c.92+1G>A	β^0	2	8,3	5	10,9
IVSI-6	HBB:c.92+6T>C	β^+	1	4,2	4	8,7
IVSII-745	HBB:c.316-106C>G	β^+	1	4,2	2	4,3
Codon 39	HBB:c.118C>T	β^0	2	8,3	2	4,3
Hb Monroe	HBB:c.92G>C	β^0	1	4,2	2	4,3
Poly A (A>G)	HBB:c.*111A>G	β^+	1	4,2	1	2,2
TOTAL			**24**	**100**	**46**	**100**

Hb – hemoglobin. * The HGVS Nomenclature is an internationally recognized standard for the description of DNA, RNA, and protein sequence variants.

3.2. Hematological Parameters and Hb Levels of β-Thalassemia Carriers

As expected, hematological and biochemical parameters of all heterozygote carriers were consistent with β-thalassemia phenotype, as described in Table 2. Overall, their mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) values were lower, while HbA2 and HbF levels were slightly elevated. Carriers of Hb Lepore-BW had significantly higher MCH values (mean=20.283±0.400 pg) compared to the carriers of other *HBB* variants (mean=18.578±0.519 pg; p=0.034, t-test). On the other hand, levels of HbA2 were lower in Hb Lepore-BW carriers (mean=2.409±0.0939%), compared to the carriers of other *HBB* variants (mean=4.841±0.136%; p<0.001, t-test). In addition, we observed a trend toward higher mean MCV value in Hb Lepore-BW carriers (mean=61.05±1.276 fl) in comparison to carriers of other β-globin gene variants (mean=57.011±1.631 fl; p=0.098, t-test).

Table 2. Hematological and biochemical features of 46 β-thalassemia patients.

Hemoglobin [g/L] (n=13), median (range)	113 (91-131)
MCV [fl] (n=15), median (range)	58.4 (51.6-68.5)
MCH [pg] (n=15), median (range)	19.3 (16.5-22)
HbA2 [%] (n=31), median (range)	4.1 (1.8-5.7)
HbF [%] (n=29), median (range)	3.3 (0.4-30.8

3.3. β-Thalassemia Carriers in Clinical Practice

Twenty-two out of 30 (73,3%) children with the confirmed β-thalassemia trait and no iron deficiency received previous oral iron therapy in appropriate dosages for a period of 3 weeks to 4 months, after which the complete blood count was repeated by the primary physician. As no improvement in Hb and red cell indices was observed, children were referred to the pediatric hematologist for further evaluation where they received advice to discontinue oral iron. The patients were also educated about importance of molecular testing on genes affecting metabolism of bilirubin, iron, and bone, such as *UGT1A1* variants, *HFE* variants, *VDR*, *COLIAI*, *COLIA2*, and *TGFB1* variants, to predict possible secondary β-thalassemia trait complications. Additionally, patients/parents were informed about the need for testing on genetic thrombophilia risk factors (*MTHFR*, Factor II, Factor V).

4. Discussion

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Beta-thalassemias are a heterogeneous group of inherited hemoglobin disorders characterized by reduced or absent β-globin chain synthesis. Historically, thalassemias have been most frequent in subtropical malaria-endemic regions of the world, reflecting the relative resistance of carriers to *Plasmodium falciparum* and higher frequency of consanguineous marriages [1,9]. Due to large-scale migrations, the prevalence of β-thalassemia is continuously increasing in non-endemic regions, including Northern and Western Europe and North America, and making this disease a global health concern [5].

A limited number of studies have reported population-based estimates of β-thalassemia, ranging from 0.2/100 000 people in Spain in the period 2014–2017 to 49.6/100 000 people in Iraq in the period 2003–2018 [10,11], and varying even within countries [12]. To better understand global β-thalassemia burden and help direct public health policies, up-to-date epidemiological data are needed for many countries. Disease-causing variants in thalassemia are often population specific. There is a particular paucity of data for Croatia. Our study presents the largest national study to date comprising a total of 46 β-thalassemia cases originating from Croatian Littoral and Istria, and could help to formulate a Croatian carrier screening program.

The molecular basis of β-thalassemias has been studied in many countries. Only 20 variants account for more than 80% of the β-thalassemia variants worldwide due to geographical clustering, where each population has a few common variants and a varying number of rare ones [13]. Hb Lepore-BW is the predominant cause of -thalassemia in Croatia with the frequency of 32,6%. The results are similar to the results of our neighboring country Serbia, with the reported incidence of Hb Lepore-BW of 26,2% [14] (pp. 477-485). This structurally abnormal form of Hb is a result of fusion of - and -globin genes, and our results demonstrated significantly lower levels of HbA2 (2 2) in Hb Lepore-BW group compared to the carriers of other HBB variants. These findings correspond to the previous studies and could be explained by decreased synthesis of -globin chain [15,16].

Most common mutations affecting *HBB* gene in our cohort, HBB:c.93-21G>A (β^+ IVS-I-110), HBB:c.315+1G>A (β^0 IVS-II-1), HBB:c.92+1G>A (β^0 IVS-I-1) and HBB:c.92+6T>C (β^+ IVS-I-6) were detected in more than 52% of all affected β-globin alleles. These results are very similar to the results of other European countries (Romania, Greece, Bulgaria, Hungary, Macedonia, and Italy). However, although frequency of the HBB:c.118C>T (codon 39) variant was relatively high in surrounding countries (Italy 44,8%; Hungary 29,4% Bulgaria 29,1%; Greece 19,51; Serbia 16,2%; Romania 16,0%), our results showed the incidence of only 4,3% probably due to genetic drift [17–22].

Five *HBB* gene variants account for approximately 85% of all β-thalassemia variants in Croatian population. These findings are in accordance with the observation that each population has a few common variants [13], that enables the choice for the population-specific targeted carrier screening methods. Although thalassemia is sporadic in Croatia, the results might provide information on the history and origin of the different β-thalassemia variants. Very similar high frequency of Hb Lepore-BW in Croatia and Serbia can be explained by the common history and possible common ancestry. The second most frequent IVSI-110 variant, previously reported to be of the Eastern Mediterranean (Turkish) origin, probably reflects historical migrations over Balkan peninsula [23]. The overall similarity of the five commonest Croatian *HBB* gene variants with those reported in other European countries can be attributed to the territorial proximity and a geographic position of Croatia at the crossroads of Central Europe, the Balkans, and the Mediterranean.

Although DNA testing for thalassemia trait is not a routine procedure, there are several reasons why genetic studies of β-thalassemia heterozygosity are important.

Unresolved laboratory hematology and implications for pediatric practice. In β-thalassemia minor, complete blood count usually shows no or mild anemia (Hb >9-10 g/d), red blood cell (RBC) count is increased or normal, and MCV and MCH decreased. Examination of the peripheral blood smear reveals microcytosis, hypochromia, and variations in RBC size and shape. The reticulocyte count is normal or slightly elevated [24]. Differential diagnosis from iron deficiency anemia (IDA) is important, foremost for the avoidance of unnecessary investigations and for the treatment planning [25,26]. The RDW (RBC Distribution Width) is elevated in more than 90% of persons with IDA, and in only 50% with heterozygous thalassemia [27]. A variety of discriminative hematological indices

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have been proposed for IDA and β-thalassemia trait, each with some degree of inaccuracy. In children, Mentzer index (MCV/RBC) can help distinguish; in IDA, the ratio is usually greater than 13, and in thalassemias less than 13, whereas a ratio of 13 is considered uncertain [27,28].

Often diagnostic confirmation of these two entities requires further tests involving serum ferritin and Hb electrophoresis. Measurement of the serum ferritin level is the most accurate test to diagnose IDA. In the absence of inflammation, a normal ferritin level (> 15 ng/ml) generally excludes iron deficiency. Hb electrophoresis in thalassemia carriers usually demonstrates reduced HbA, increased levels of HbA_2 (> 3.5% of total Hb) and increased HbF (>1%). A high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) are two common techniques used for quantifying HbA_2. However, a normal concentration of HbA_2 does not rule out β-thalassemia trait, especially if there is concomitant iron deficiency, which can lower HbA_2 levels into the normal range. Besides, borderline HbA_2 values may occur as a consequence of mild/silent *HBB* mutations and co-inherited β-thalassemia and α- or δ-thalassemia. As conventional techniques may not be reliable, only confirmation with molecular genetic testing provides accurate diagnosis [29–31].

Many β-thalassemia carriers are erroneously believed to have IDA. In our study, 22 out of 30 (73,3%) children with the confirmed β-thalassemia trait received previous oral iron therapy with no improvement. It is important to remember that children with thalassemia trait-related anemia should not take iron supplements unless they have concomitant iron deficiency. However, several studies reported an underestimation of the coexistence of iron deficiency and thalassemia trait in children [32–34]. This coexistence should not be neglected, and iron therapy should be administered in iron deficient children. We propose that if Hb < 11 g/dL in a case of thalassemia minor, one should screen for iron deficiency simultaneously.

Genetic counselling. Severe forms of thalassemia rarely escape from clinical diagnosis. Beta-thalassemia minor is the heterozygous state that is usually asymptomatic and can be easily dismissed. Carriers are frequently unaware of their disorder. As a rule, thalassemia trait is identified during the screening because of an affected family member, or rarely incidentally during routine laboratory analysis, e.g., HBA1c values in diabetic patients [35]. Molecular analysis is the only definitive way to diagnose heterozygous thalassemia and can be helpful in qualifying which HBB variant families harbor.

Genetic counselling is inseparable from genetic diagnosis, allowing couples at risk to make informed decisions on their reproductive choices. Extreme phenotypic and molecular heterogeneity of β-thalassemia and potential co-inheritance of various abnormal Hb require experienced genetic counselor. Simplifying complex information, if one partner is a known carrier and planning to start a family, it is advisable for another partner to be tested as well. Thalassemias are inherited in an autosomal recessive manner. Therefore, through genetic counselling, ideally in the pre-conception period or as early as possible in the pregnancy, and with the possibility of prenatal diagnosis, the birth of a child with thalassemia major can be avoided, if desired.

Genetic testing improves the healthcare of adult β-thalassemia carriers. The timely carrier screening can be carried out for the direct benefit of adult patients. It is well documented that the remarkable phenotypic diversity of β-thalassemia individuals is associated with a great genotype variety. The primary genetic determinants are mostly different types of *HBB* gene mutations ($\beta^0/\beta^+/\beta^{++}$) leading to decreased or absent production of β-globin chains. However, the causal relationship between phenotype and genotype might be further complicated by the interaction of secondary and tertiary genetic modifiers. Two important secondary modifiers - co-inheritance of α-thalassemia and variants associated with increased HbF synthesis - have emerged, but they do not explain all clinical heterogeneity [36]. The genes involved are *HBA*, *HBG*, *BCL11A*, *HBS1L-MYB* and other cofactor genes regulating erythropoiesis [37]. Recent studies revealed that other genetic modifiers, not affecting globin imbalance directly, might moderate secondary manifestations of heterozygous β-thalassemia and response to therapies. Among these, one of the best delineated are those affecting metabolism of bilirubin, iron, and bone. UDP-glucuronosyltransferase (*UGT1A1*) gene variants (Gilbert syndrome) predispose to jaundice and the formation of gallstones. *HFE* C282Y variant, which causes the common type of hereditary hemochromatosis, might be involved in determining the variability of

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iron overload in patients with thalassemia intermedia. Homozygosity for H63D variant in *HFE* gene, when coinherited with heterozygous β-thalassemia, seems to increase iron overload. Furthermore, genetic predisposition to osteoporosis (*VDR*, *COLIAI*, *COLIA2*, and *TGFB1* gene variants) can affect thalassemia trait complications. An increased risk of thrombosis related to Factor II, Factor V and *MTHFR* gene variants and cardiac complications related to *GSTM1* haplotype, *ApoE* ε4 allele and some HLA haplotypes, have been reported in patient with thalassemia major [38–40] (pp. 339-344). Thus, in the era of molecular medicine, β-thalassemia carriers have a unique opportunity for additional genetic testing and secondary prevention strategy [37,39,41].

Besides, carrier women of childbearing age should be aware of their diagnosis. During pregnancy, the anemia of thalassemia trait often becomes more severe. Consequently, pregnant women with thalassemia trait would have a higher risk of adverse pregnancy outcomes compared to pregnant women without thalassemia, and higher level of prenatal care and consultations between obstetricians and hematologists should be considered [42]. Transfusions are rarely necessary, but adequate iron and folate supplementation is recommended to avoid compounding the causes of anemia [24].

5. Conclusions

Our results confirm that the accurate diagnosis of heterozygous thalassemia is based on molecular genetic testing. Our study identified the spectrum of β-thalassemia variants in Croatia. We believe it is crucial to investigate the population molecular characteristics of thalassemias for effective targeted genetic screening and counseling. More importantly, additional variants in known modifier genes of β-thalassemia should be considered in a follow-up of carriers due to possible secondary complications. Pediatrician's recommendations for genetic testing and potential special treatment are required. This study provides important new insights into the relevance of β-thalassemia heterozygosity in a pediatric clinical practice in Croatia and globally.

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