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Original paper

RAPID CHARACTERIZATION OF β-THALASSEMIA MUTATIONS BY REVERSE DOT BLOT AND ALLELE-SPECIFIC PCR ANALYSIS

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Summary: This paper reports a case of β-thalassaemia major whose molecular diagnosis was achieved by using modern methods of molecular genetics. This example demonstrates the strategy we chose to detect β-thalassaemia mutations in the Republic of Serbia in order to complete molecular screening in our population and to make prenatal diagnosis in pregnancies at risk. The analysis of genomic DNA isolated from the blood of patient affected with thalassaemia major is carried out by the methods: RDB (reverse dot blot) and ARMS (amplification refractory mutation system). It is shown that the patient is a compound heterozygote for two β--thalassaemic mutations: β^+IVSI-110 and β^+IVSI-6.

Key words: β-thalassaemia, molecular diagnosis.

Introduction

Thalassaemia syndromes are a group of hereditary disorders in which a defect in the synthesis of globin polypeptide chains of haemoglobin is present. Clinical manifestations are diverse, ranging from asymptomatic hypochromia and microcytosis to profound anaemia, which is fatal *in utero* or in early childhood if untreated (thalassaemia major).

Taken as a group, thalassaemia syndromes are the most common single gene disorder known. They are most common in the Mediterranean basin and equatorial regions of Asia and Africa. Republic of Serbia belongs to the »thalassemia belt« where gene frequencies are very high (1). In former Yugoslavia 8 of 10 analyzed thalassaemic alleles were of the β-thalassemia type (2). Efremov (3) has reported that the average incidence of beta-thalassaemia trait in former Yugoslavia was 1.2%, ranging from 2.9% in the south (Macedonia) to 0.8% in the northwest (Croatia). In the population study of the haemoglobinopathies, Beksedic et al. (4) have determined that the prevalence of β-thalassaemia in the Republic of Serbia is 1.9 %.

Most types of β-thalassaemia are due to point mutations (1). It has been determined that 5 or 6 mutations usually account for more than 90% of the cases of β-thalassaemia in a given ethnic group or geographic area (5, 6). These data have been considered for the design of the strategy for molecular screening of β-thalassaemia mutations in the Republic of Serbia. Screening is carried out by PCR based methods: RDB (reverse dot blot) and ARMS (amplification refractory mutation system). The set of RDB probes and ARMS primers are complementary to the most common mutations in the Mediterranean area (7 9). If the mutation is not determined by these methods, direct sequencing analysis is to be performed.

This paper reports a case of β-thalassaemia major whose molecular diagnosis was accomplished by using simple sensitive and rapid methods (RDB and ARMS). It is shown that the patient is a compound heterozygote for two common β-thalassemic mutations in the Mediterranean area.

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Methods

Case history

Basic haematologic data, HbF (collected by standard methods) (10, 11) and HbA_2 (estimated by elution from celogel electrophoretic strips) showed that the patient had typical features of a β-thalassaemia major. The now 19-year old male patient requires permanent blood transfusion therapy.

DNA methods

DNA was extracted from leukocytes by standard techniques (12).

Reverse dot blot analysis

Screening for known β-thalassaemia mutations was performed by reverse dot blot analysis. The method is non-radioactive and is based on e hybridization of the allele specific oligonucleotide (ASO) probes and the amplified β-globin DNA labeled by biotine. ASO probes complementary to the most common β-thalassaemia defects in the Mediterranean area were immobilized onto a nylon membrane (Pall Byodine C). Amplification of β-globin gene was obtained from the position 158 5' to the cap site to the position 60, 3' to the polyadenylation signal by PCR, as previously described (13). The sequences of ASO probes, as well as the hybridization conditions used for the assays were as in Saiki et al. (7).

ARMS analysis

The basis of this method is the observation that oligonucleotides that are complementary to a given DNA sequence, except for a mismatch at their 3'-OH residue, will not function as primers in PCR under appropriate conditions. A typical ARMS test consists of two complementary reactions. The first reaction contains a primer specific for the normal sequence, the second reaction contains a mutant-specific primer. A normal individual generates PCR products only in the »normal reaction«; heterozygote gives products in both reactions, and a homozygote mutant individual does so only in the »mutant reaction«. The PCR conditions were as previously described, except the modifications in the quantities of primers and the magnesium chloride concentration (8, 9). The primers for ARMS analysis of the mutation β^+IVSI-110 were as follows: common primer: 5'-ACCTCACCCTGTGGAGCCAC-3'; mutant-specific primer (M): 5'-ACCAGCAGCCTAAGGGTGGGAAAATACACT-3'; primer specific for the normal sequence (N): 5'-ACCAGCAGCCTAAGGGTGGGAAAATACACC-3'.

The primers for ARMS analysis of the mutation β^+IVSI-6 were as follows: common primer: 5'-ACCTCACCCTGTGGAGCCAC-3'; mutant-specific primer (M):

5'-TCTCCTTAAACCTGTCTTGTAACCTTCATG-3'; primer specific for the normal sequence (N): 5'-TCTCCTTAAACCTGTCTTGTAACCTTCATA -3'.

Results and Discussion

RDB analysis of β-globin gene of the patient indicated that the patient is a compound heterozygote for two common mutations in the Mediterranean area (β^+IVSI-110 and β^+IVSI-6). Amplified DNA did not hybridize to the probes for the other point mutations representing molecular defects in β-globin gene detected in our population The hybridization to βn probe is used as the membrane and hybridization control. (*Figure 1*).

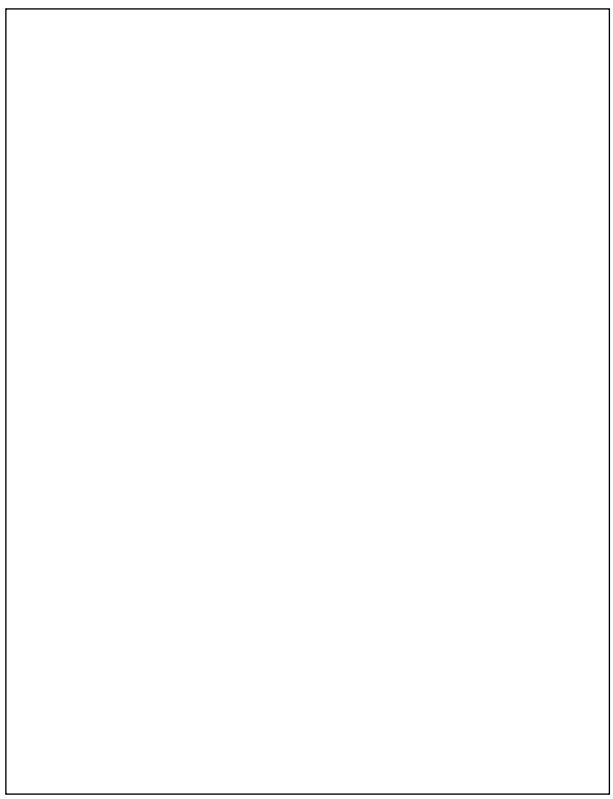

Figure 1 Reverse dot blot analysis with oligonucleotide probes complementary to: the normal β-globin gene at the position 110 in the first intron (βn), β^+IVSI-110 mutation (βI-110), $\beta^0$39 mutation (β39), β^+IVSI-6 mutation (βI-6), β^0IVSI-1 mutation (βI-1), β^0IVSII-1 mutation (βII-1) and β^+-87 mutation (β-87). Hybridization with amplified DNA from leucocytes of the patient revealed that he is a compound heterozygote for the mutations β^+IVSI-110 and β^+IVSI-6. The detection of hybridization is based on the interaction of biotine (patient's DNA amplified by PCR) and streptavidine-horse radish peroxidase used as the indicator of biotine.

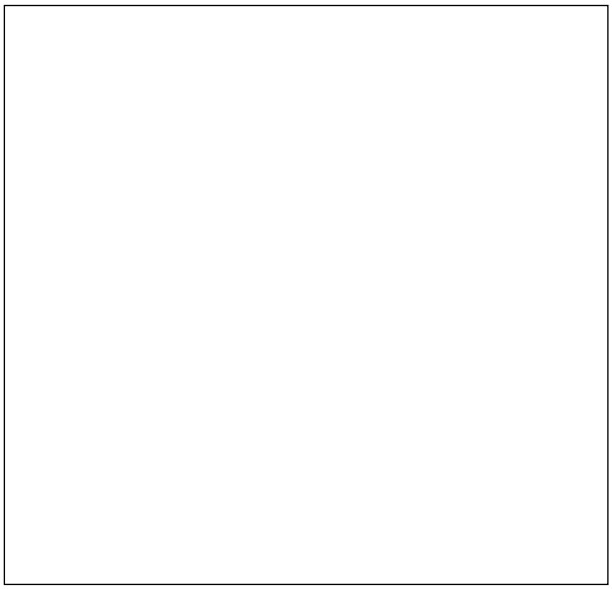

Figure 2 ARMS analysis of patient's β-globin gene for the β+IVSI-110 and β+ IVSI-6 mutations. ARMS reactions (M with mutant specific primers and N with primers specific for the normal sequence) confirm that the patient is a compound heterozygote both for the mutations β+IVSI-110 (390 bp PCR fragments) and β+IVSI-6 (286 bp PCR fragments). 861 bp PCR fragments are the positive control of the PCR reaction.

ARMS analysis confirmed that the patient is a carrier of both (β+IVSI-110 and β+IVSI-6) mutations (*Figure 2*).

The β+IVSI-110 is the first base substitution (G → A) identified in a β-thalassaemia gene (14, 15). It creates new alternative splice site in the first intron of β-globin gene. The other mutation (β+IVSI-6 (T → C)) is the mutation of splice site consensus sequence that partially blocks normal splicing (16). These mutations have been shown to be very common form of β-thalassaemia in the Mediterranean (1). The compound heterozygosity for these mutations shows the phenotype of β-thalassaemia major.

Our results show that the molecular diagnosis of the specific β-thalassaemia mutations may be accomplished reliably by simple RDB analysis and confirmed by ARMS. If the mutation is not determined by these methods, direct sequencing analysis is to be performed. The main advantages of this strategy are simplicity, sensitivity and rapidity. A similar approach to that described here has been applied in Italy (17), south China (18) and Greece (19). Better definition of the relative prevalence of β-thalassaemia mutations in Serbian at risk population has improved this strategy for screening prospective parents and making prenatal diagnosis.

BRZA KARAKTERIZACIJA β-TALASEMIJSKIH MUTACIJA METODAMA REVERZNOG DOT BLOTA I ALEL-SPECIFIČNOG PCR-a

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Kratak sadržaj: U ovom radu je prikazan slučaj β-talasemije major čija je dijagnoza postavljena na molekularnom nivou korišćenjem modernih metoda molekularne genetike. Ovaj primer ilustruje strategiju koju smo izabrali da bi se detektovale β-talasemijske mutacije u R Srbiji, a u cilju sprovođenja skrininga u našoj populaciji prenatalne dijagnostike u rizičnim porodicama. Za analizu genomske DNK izolovane iz krvi pacijenta sa kliničkom slikom talasemije major, korišćene su metode reverznog dot blota (RDB) i alel-specifičnog PCR-a (ARMS). Pokazano je da je pacijent dvostruki heterozigot za β-talasemijske mutacije: β+IVSI-110 i β+IVSI-6.

Ključne reči: β-talasemija, molekularna dijagnostika.

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