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Comparison of HPLC results of patients with hemoglobin D with DNA sequence analysis: Detection of compound heterozygosity HbD/β-Thalassemia traits

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Abstract

Objective: In our study, we aimed to compare the results of patients with near-total hemoglobin D variants detected by the HPLC method with DNA sequence analysis.

Materials and methods: In our laboratory, hemoglobin variant analysis was performed with an HPLC analyzer (Adams A1C HA8180T, Arkray, Inc., Kyoto, Japan) from EDTA-tubed whole blood samples of three male patients. PCR-DNA Sanger sequence analysis was then performed on these samples for a definitive diagnosis.

Results: We found that three male patients had hemoglobin D variants close to the total by HPLC method (HbD levels are 97.92%, 93.2%, and 97.6%, and HbA levels are 0% for all patients, respectively). In two patients, we did not detect hemoglobin A2 levels by HPLC, while in one patient, we detected <3.5% HbA2. According to the results of PCR-DNA sequence analysis, we found that all three patients had heterozygous Hemoglobin D, characterized by the p.Glu121Gln (c.364G>C) mutation. In addition, the patients had c.92 +5G>C, IVS2-1G>A (c.315+1G>A), and p.Lys9Valfs*14(c.25_26del) mutations, which were pathologically identified and consistent with heterozygous β-thalassemia carriage. Thus, we found that three patients had compound heterozygous HbD/β-thalassemia.

Conclusion: The presence of hemoglobin D is close to total hemoglobin D in percentage, according to the HPLC result, but does not always indicate homozygous hemoglobin DD. In addition, β-thalassemia carriage may be missed from the laboratory results of those who are carriers of the hemoglobin D variant and have nonincreasing hemoglobin A2 levels. In these patients, it should be clarified whether they are β-thalassemia carriers by genetic methods. In this way, the birth of babies with β-thalassemia major can be prevented.

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Introduction

Structural changes or synthesis disorders in the polypeptide chains of the hemoglobin molecule cause hemoglobinopathies. Hemoglobinopathies are classified into two main groups: abnormal hemoglobins, and thalassemias (1). Hemoglobin D, which is an abnormal hemoglobin, is one of the most common hemoglobin variants worldwide. Studies conducted in our country show that it is the second most common variant after hemoglobin S. In addition, hemoglobin D is also called hemoglobin D-Los Angeles and hemoglobin D-Punjab in the literature (2). Hemoglobin D is a variant resulting from the substitution of glutamine for glutamate, the 121st amino acid in the β-globin chain. Homozygous hemoglobin DD disease is rare and usually asymptomatic, but sometimes mild to moderate hemolytic anemia may develop in these patients (3).

Methods such as high-pressure liquid chromatography (HPLC), capillary electrophoresis, electrophoresis, and electrophoresis are used to screen hemoglobinopathies such as hemoglobin D. However, definitive diagnosis is made by genetic methods. HPLC is a separation technique based on the interactions of the components in a mixture with the stationary phase and the mobile phase moving above the stationary phase along this phase. In cation exchange HPLC, hemoglobin binds to the negatively charged stationary phase and are then separated by the addition of a positively charged mobile phase that competes with hemoglobin for binding to this phase. Hemoglobin is separated at a specific rate according to their affinity for the stationary phase. The amount of hemoglobin is calculated as a percentage of the area under each peak in the graph obtained from the measurement (2).

β-thalassemia minor is characterized by a hereditary decrease in β-globin synthesis. The β-globin gene is located on the short arm of chromosome 11 (11p15.5) within the β-globin gene cluster. Heterozygotes (carriers) are often referred to as having β-thalassemia minor. β-Thalassemia minor is characterized by reduced MCV and MCH, with increased Hb A2 level (4). Hemoglobin D and β-thalassemia combined heterozygosity has been reported in the literature. In these patients, β-thalassemia gene mutations have been defined in addition to the presence of hemoglobin D close to total in percentage (5-8). However, in cases of only hemoglobin D carriage without β-thalassemia minor, the presence of hemoglobin D with a rate of 35-45% and the presence of HbA with a rate of more than 50% have been shown (2).

DNA sequence analysis is used in the definitive diagnosis of hemoglobinopathies at the gene level. There are many different methods for this analysis, including Gap-PCR, MLPA (ligation-dependent multiple probe amplification), DNA microarray, and Sanger sequencing (9). The Sanger sequencing method is based on enzymatic DNA synthesis and is the most widely used sequence analysis technique. In the method, the DNA strand whose sequence is to be determined is used as a template for the new strand to be synthesized. The method is based on the fact that DNA polymerase can use dNTP (deoxyribonucleoside triphosphate) and ddNTP (dideoxyribonucleoside triphosphate) as substrates (10).

In our study, we aimed to compare the results of three patients with near-total hemoglobin D variants detected by HPLC in our laboratory with the results of DNA sequence analysis.

Materials and methods

The study was approved by the University of Health Science Clinical Research Ethics committee (no: 19/24, date: 03.11.2023).

For the study, three random patients with Hb D variants close to the total in percentage according to the HPLC result in our laboratory in November 2023 were included in the study. In our laboratory, hemoglobin variant analysis was performed with an HPLC device (Adams A1C HA8180T, Arkray, Inc., Kyoto, Japan) from whole blood samples of three male patients in an EDTA tube. Subsequently, PCR-DNA Sanger sequence analysis was performed in the genetics laboratory for definitive diagnosis after obtaining informed consent from the patients. Data from three patients were obtained from the laboratory information system. Patients with hemoglobin D levels between 30-45% and also patients with HbA levels of more than 50% were excluded from the study.

Results

We found that three male patients had a hemoglobin D variant close to the total in percentage by HPLC method (Table 1).

Patient 1 was a 35-year-old man who presented to

family physician for pre-marital screening. He had no anemia on complete blood count (hemoglobin: 136 g/L), but MCV and MCH values were below the reference range (MCV: 59.8 fL, MCH: 20.2 pg). In hemoglobin variant analysis, HbF was 2.1%, and HbD level was 97.92%. HbA and HbA2 were not detected at all in the analysis (Table 1).

Table 1: Demographic and laboratory data of the patients

Patients	Pre-diagnosis	Age	Gender	HbD %	HbA %	HbA_2 %	HBF %	RBC (10^6/uL)	Hb (g/L)	MCV (fL)	MCH (pg)
Patient 1	Pre-marital screening test	35	Male	97.92	0	0	2.1	6.73	136	59.8	20.2
Patient 2	Pre-marital screening test	27	Male	93.2	0	3.4	3.4	6.37	126	58.1	19.8
Patient 3	Variant analysis in patient with undetectable HbA1c	72	Male	97.6	0	0	2.4	6.02	130	61.2	20.1

Hb: Hemoglobin, RBC: Red blood cell, MCH: Mean corpuscular hemoglobin, MCV: Mean corpuscular volume.

Patient 2 was a 27-year-old male patient who presented to the family physician for pre-marital screening. He had no anemia on complete blood count (hemoglobin: 126 g/L), but MCV and MCH values were below the reference range (MCV: 58.1 fL, MCH: 19.8 pg). Hemoglobin variant analysis showed HbF: 3.4%, HbA2: 3.4%, HbD: 93.2% (Table 1).

Patient 3 was a 72-year-old male patient who presented to a family physician for routine HbA1c measurement. The patient's HbA1c level could not be detected by HPLC. We obtained the same result in the repeated measurement, and hemoglobin variant analysis was performed because it may cause interference. As a result of variant analysis, HbF was measured as 2.4% and HbD as 97.6%. HbA and HbA2 levels could not be determined (Table 1). Complete blood count showed no anemia (hemoglobin: 130 g/L), but MCV and MCH values were below the reference range (MCV: 61.2 fL, MCH: 20.1 pg).

According to the results of PCR-DNA sequence analysis, we found that all three patients were heterozygous Hemoglobin D carriers characterized by p.Glu122Gln (c.364G>C) mutation (Table 2).

In addition, the patients had c.92+5G>C, IVS2-1G>A (c.315+1G>A), and p.Lys9Valfs*14(c.25_26del) mutations, and these mutations were consistent with heterozygous β-thalassemia carriage (Table 2).

Table 2: DNA Sequence Analysis Results of Patients

	Mutation results for HbD	Mutation results for β-thalessemia
Patient 1	p.Glu121Gln(c.364G>C) Heterozygous	c.92+5G>C Heterozygous
Patient 2	p.Glu121Gln(c.364G>C) Heterozygous	IVS2-1G>A (c.315+1G>A) Heterozygous
Patient 3	p.Glu121Gln(c.364G>C) Heterozygous	p.Lys9Valfs*14(c.25_26del) Heterozygous

Discussion

Hemoglobin D is the second most common hemoglobin variant in our country and is formed when glutamine replaces glutamate at position 121 in the β-globin chain. Although methods

such as HPLC and electrophoresis are used in the screening of hemoglobinopathies such as hemoglobin D, these are screening methods, and the definitive diagnosis is made using genetic methods (2). In our study, we compared the results of patients in whom we detected a hemoglobin D variant close to the total by HPLC with the results of DNA sequence analysis.

Based on the major findings of the study, three male patients had a hemoglobin D variant close to the total percentage using the HPLC method. This suggested homozygous Hb DD. According to the results of PCR-DNA sequence analysis, we found that all three patients had heterozygous Hemoglobin D characterized by p.Glu121Gln (c.364G>C) mutation. The patients had c.92+5G>C, IVS2-1G>A (c.315+1G>A), and p.Lys9Valfs*14(c.25_26del) mutations, and these mutations were pathologic and consistent with heterozygous β-thalassemia carriage. In other words, we found that three patients were combined heterozygous hemoglobin D carriers and β-thalassemia carriers.

A misdiagnosis of β-thalassemia carrier in samples with HbD can be a cause of inappropriate genetic counseling, thus having a new case of β-thalassemia major. In HbD/ β-thalassemia patients with combined heterozygosity, nonincreasing hemoglobin A2 levels may be missed from β-thalassemia carrier laboratory results. These patients may be considered as not having β-thalassemia carriage because the HbA2 level is used as a diagnostic marker for β-thalassemia trait. Therefore, patients with near-total hemoglobin D should be examined by genetic methods to determine whether they are carriers of β-thalassemia even if their HbA2 levels are not high. It should also be clarified whether these patients are homozygous or heterozygous HbD carriers. This will shed light on the evaluation of future family members in terms of hematologic diseases.

In the literature, we reviewed the results of studies in which there were combined heterozygous cases for hemoglobin D carrying and β-thalassemia minor. Upon examining the studies, Panyasai et al. (5) indicated that in study number four, a seven-year-old girl exhibited 80.7% HbD and 3.2% HbA2, as per the HPLC results. However, as a result of this genetic analysis, they found that this child had compound heterozygosity HbD-Punjab/ β-thalassemia.

Taghavi et al. (6) reported the result of three cases referred that had a combination of β-thalassemia and Hb D traits (Hb D: 94.2%, 91%, 94.5%, respectively, for three cases). They reported that in all three cases, the Hb D level was elevated, and no HbA was detected electrophoretically. HbA2 levels were elevated in all cases (between 4.0 and 5.6%). They initially thought that all cases were homozygous for Hb D according to the electrophoresis pattern. Then, they performed genetic analysis, and they reported that in all cases, the mutations in the β-globin gene were detected by the ARMS-PCR technique. In their first case (HbD: 94.2%), the HPLC results of the patient's parents were available. The father of this case was shown to be a β-thalassemia carrier (HbA2: 6%), while the mother was an HbD carrier (HbD: 40.6% without high HbA2). In other words, in the case of combined heterozygosity, the cases had genetically inherited HbD or β-thalassemia carriage from their parents. In these cases, it seems that the β-globin chain is produced from a chromosome that carries both the HbD variant and the other alleles that are responsible for the mutation (IVSII-1 and IVSI-5) without production; therefore, in Hb electrophoresis, no normal Hb A was detected.

Denic et al. (7) showed in their study that six-month-old infants of a HbD carrier mother (HbD: 35.2%) and a β-thalassemia carrier father (HbA: 94.3%, HbA2: 5.2%) had compound heterozygosity HbD/β-thalassemia (Hb D: 88.5%, Hb A2: 6.3%). However, the other two children of this family were the only carriers of HbD (HbD: 35.8% and 39.0% without high HbA2, respectively, for each child).

When the results of the studies are examined, HbA2 levels did not increase in some of the compound heterozygous HbD/ β-thalassemia cases (5, 11), while HbA2 levels were found above the cut-off value in others (6, 7, 12). The differences in methods between the studies are also noteworthy. In some studies, HbA2 quantification was performed by HPLC (5, 7, 11, 12), and in one study, it was performed by electrophoresis (6). Panyasai et al. (5) compared the HbA2 results in their study and also compared the HPLC result with the capillary electrophoresis result. Although they found normal HbA2 levels as a result of HPLC, they found increased HbA2 levels of 7.2% in a girl with compound heterozygosity HbD-Punjab/ β-thalassemia as a result of capillary electrophoresis. The researchers stated that the capillary electrophoresis method may be more useful in the detection of compound heterozygosity HbA2. In our study, we found HbA2 levels within

the reference range in one patient, but we could not detect HbA2 in two patients. HbD-Punjab elute close to HbA2 on HPLC, and they generally lead to underestimated HbA2 and elevated HbF levels, but these hemoglobinopathies migrate separately from HbA2 on capillary electrophoresis (13, 14). Therefore, HbA2 measured by column chromatography or HPLC is not a reliable parameter for differentiating the homozygote of HbD-Punjab from the compound heterozygote of these HbD-Punjab with β-thalassemia. Capillary electrophoresis can be used as an additional method for HbA2 quantitation in these patients. However, genetic methods are still required for definitive diagnosis.

Also in the literature, in a Saudi family with nine children, the father is compound heterozygous for hemoglobin HbD-Punjab/β-thalassemia, the mother is a carrier for β-thalassemia, and three of their children are transfusion dependent β-thalassemia. Two of the children are compound heterozygous for Hb D Punjab/β-thalassemia like the father but with different genotypes. The other two children have HbD-Punjab traits, while two other children have β-thalassemia traits (15).

Compound heterozygosity for Hb D/β-thalassemia must be carefully differentiated from homozygous HbDD in pre-marital screening. This is essential when the partner is a carrier of β-thalassemia trait. Misdiagnosis, especially during pre-marital screening, can lead to having a child with β-thalassemia major, as reported in studies (15, 16). As with numerous other countries in the Mediterranean region, thalassemia is a health problem in Turkey. In Turkey, a pre-marital screening program for the prevention of β-thalassemia major has been in effect since 2003. Prior to marriage, couples are tested to determine if they are carriers of the β-globin gene mutation or not. For those who are carriers, prenatal diagnosis is offered. In pre-marital screening, molecular testing is often unavailable, and diagnosis (and marriage guidance) often relies on hemoglobin analysis, family studies, and epidemiological facts (17).

Our study has some limitations. In our study, we did not perform HPLC and molecular analyses for hemoglobin variant analysis of the parents of our patients, and we did not repeat the HPLC results with capillary electrophoresis. However, our study results will shed light on future studies and contribute to the literature.

Conclusions

The presence of hemoglobin D is close to total hemoglobin D in percentage, according to the HPLC result, but does not always indicate homozygous hemoglobin DD. In addition, β-thalassemia carriage may be missed from the laboratory results of those who are carriers of the hemoglobin D variant and have nonincreasing hemoglobin A2 levels. In these patients, it should be clarified whether they are β-thalassemia carriers by genetic methods. In this way, the birth of babies with β-thalassemia major can be prevented.

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Informed consent: Informed consent was obtained from all subjects involved in the study.

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Contributions

Research concept and design: AA, OD, BOI, EV

Data analysis and interpretation: AA, OD, MS, HD

Collection and/or assembly of data: AA, OD, LY, BOI, EV

Writing the article: AA, OD, LY, MS, HD, BOI, EV

Critical revision of the article: AA, OD, MS, HD

Final approval of the article: AA, OD, LY, BOI, EV, MS, HD

All authors read and approved the final version of the manuscript.

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