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Chromatographic and hematological comparison of sickle cell carriers and hemoglobin D los angeles carriers

Süheyl Uçucu[1], Talha Karabıyık[2]
[1]Department of Medical Biochemistry, Muğla Public Health Care Laboratory, Muğla
[2]Department of Medical Biochemistry, Bursa City Hospital, Bursa, Turkey

Abstract
Aim: Sickle cell trait and Hb D-Los Angeles (β121Glu→Gln) variant have complete blood count (CBC) and chromatographic similarities. Therefore, the differential diagnosis of these hemoglobinopathies is problematic. The aim of this study was to differentiate hemogram results between patients diagnosed with Hb S carrier and Hb D Los Angeles carrier, to compare with HPLC thalassemia screening tests hematologically and chromatographically, and to discuss their distinctive features.
Material and Methods: The study included 128 Hemoglobin D Los Angeles and 110 Hemoglobin S carriers. Age, sex, RBC, Hb, HTC, MCV, MCH, RDW, serum iron, TIBC, ferritin, HbA2, HbF, HbA0, abnormal peak retention time (RT) and area under the peak were examined.
Results: A statistically significant difference was detected in serum RBC levels, abnormal hemoglobin, retention time, and Hb A2 levels ($p=0.036$, $p<0.001$, $p<0.001$, $p<0.001$, respectively) between Hb D Los Angeles and Hb S carriers, while levels of abnormal hemoglobin in Hb S and Hb D Los Angeles carriers were between 24.6% and 44.6% in Hb S carriers, it was between 32.2-43% in Hb D carriers. The average RT levels were 1.02 in HbS, and 0.96 in Hb D. Hb A2 levels were significantly higher in Hb S carriers.
Discussion: Although the Hb D-Los Angeles variant and the Hb S carrier profile have similar CBC and electrophoretogram, they can be distinguished from each other using RT, HbA2, and abnormal hemoglobin levels. RT and HbA2 levels seem to be the main discrimination factors, but it should not be forgotten that they do not always have sufficient discrimination power. Molecular diagnostic methods such as PCR or DNA sequencing should be used for definitive diagnosis.

Keywords
Hemoglobinopathies, Hb D-Los Angeles, Sickle Cell, Hb S, HPLC

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Corresponding Author: Süheyl Uçucu, Department of Medical Biochemistry, Muğla Public Health Care Laboratory, Muğla, Turkey.
E-mail: suheyllucucu@gmail.com P: +90 555 306 28 74
Corresponding Author ORCID ID: https://orcid.org/0000-0002-6320-2457

Introduction

Hemoglobinopathies affect millions of people around the world. It causes the birth of hundreds of thousands of babies with hemoglobin disorders and the deaths of hundreds of thousands of babies worldwide each year [1, 2].

Even though it is endemic in regions such as Sub-Saharan Africa, Asia, Mediterranean Countries, and the US in general, its prevalence is increasing even in Northern European countries where it is not commonly seen [2]. Migrations that have occurred alter the gene pool [3, 4]. Mutations are transmitted to different populations by genetic drift. Therefore, new variants continue to be added to the gene pool each year [5, 6].

Turkey is a region where many civilizations were founded, and the genetic variability is abundant. For this reason, the diversity and frequency of hemoglobinopathy have increased concordantly. Rare variants in different geographies have been detected [6]. Variant types and prevalence vary widely in Turkey by region. However, the most common hemoglobin variants in the world and in Turkey are sickle cell carriage (Hb S (β6Glu→Val)) and Hb D-Los Angeles (β121Glu→Gln).

In sickle cell trait, valine amino acid replaces glutamic acid due to a point mutation at codon 6 of beta globin gene on chromosome 11 [2, 7]. As a result of this point mutation, Hb S which polymerizes under conditions such as pH change, deoxygenation and dehydration and causes sickling of erythrocytes, is formed. According to the data of WHO, while Africa is the most common place in the world, it is the Mersin-Hatay region in our country. Its prevalence varies between 0.3% and 44% [3, 8]. Even though sickle cell carriage is generally known to be harmless, it is now known that it can lead to complications and may be associated with conditions such as kidney diseases, thrombosis, stroke, arthritis, pain crises, spleen sequestration, and shock [9-11]. In addition, a genetic modifying factor that may coexist with Hb S may also lead to a very severe course of the phenotype [9].

While Hb D Los Angeles being Hb A/D heterozygous or Hb D/D homozygous does not lead to a clinical phenotype, simultaneous inheritance with different variants can lead to a variable spectrum of clinical conditions from mild to severe [12, 13].

These two variants, which affect millions of people around the world, are similar in terms of hematological (CBC), alkaline electrophoretic mobility, concentration and HPLC profiles. Differential diagnosis of these hemoglobinopathies is therefore problematic [2, 7].

In this study, hemogram results of patients diagnosed with Hb S carrier and Hb D Los Angeles carrier will be compared with HPLC thalassemia screening tests hematologically and chromatographically and their distinctive features will be discussed.

Material and Methods

This study was carried out retrospectively between 01.01.2015 - 01.06.2021. Ninety (38%) women and 148 (62%) men aged 11-67 years who applied to the Thalassemia Diagnosis, Treatment and Research Center were included in the study. Patients included in the study did not have hypothyroidism problems, iron deficiency anemia, nutritional disorder or alpha thalassemia suspicion, kidney diseases, thrombosis, stroke, arthritis and shock.

Ethical approval was obtained from the MSKÜEAH (Muğla Sıtkı Koçman University Training and Research Hospital) Research and Ethics Committee (registration no. 111). The study was conducted in accordance with the Helsinki Declaration principles.

The study included 128 Hemoglobin D Los Angeles and 110 Hemoglobin S carriers. Age, sex, RBC, Hb, HTC, MCV, MCH, RDW, serum iron, TIBC, ferritin, HbA2, HbF, HbA0, abnormal peak retention time (RT) and the area under the peak were examined. Complete blood cell parameters and indices were determined using Sysmex XN 1100 (Sysmex Diagnostic, Japan). Hemoglobin variant analysis was performed using the Primus Ultra II device (Trinity Biotech Diagnostic, Ireland) which is based on high-pressure liquid chromatography (HPLC). Serum iron, TIBC levels were measured by Photometric Method in the Cobas 501 (Roche Diagnostics, Germany) analyzer. Ferritin levels were measured by ECLIA Method and in Cobas 501 (Roche Diagnostics, Germany) device.

Statistical analysis

Jamovi 1.6.15 program was used for statistical analysis. The Shapiro-Wilk test was used to determine the data's conformity to the normal distribution. Independent T-test was used to evaluate the normally distributed variables. The Mann-Whitney U test was used for the evaluation of the variables that did not fit the normal distribution. The P-value < 0.05 was accepted as statically significant.

Results

The sociodemographic data of the patients and the minimum and maximum values of the tests are presented in Table 1. There was a statistically significant difference in serum RBC levels, abnormal hemoglobin, retention time, and Hb A2 levels (p=0.036, p<0.001, p<0.001, p<0.001, respectively) between Hb D-Los Angeles carriers and Hb S carriers.

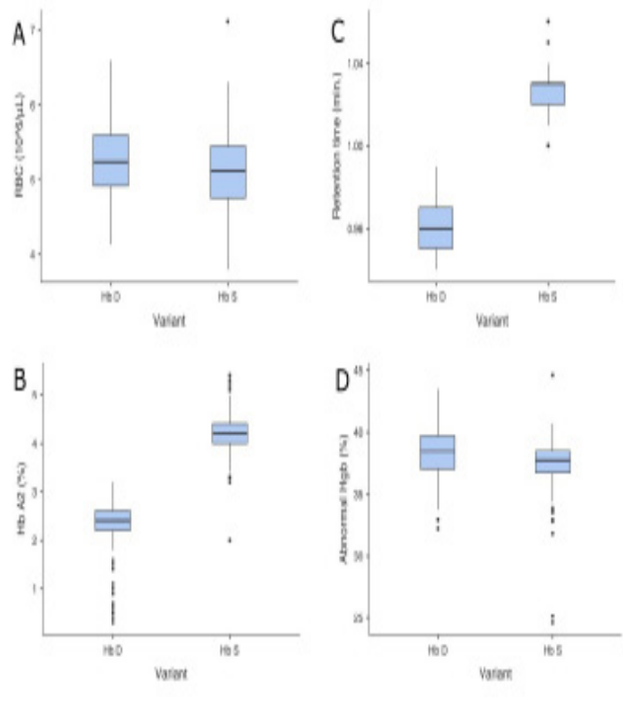

Figure 1. Box plots of the Sickle Cell Carriers and Hb D Los Angeles Carriers Groups

Table 1. Characteristics and Hematological Parameters Among Sickle Cell Carriers and Hb D Los Angeles Carriers Group (n=238)

	Sickle Cell Carriers (n=109)		Hb D Los Angeles C (n=129)		P*
	Mean±SD	Min-max	Mean±SD	Min-max	
Age (years)	30.9±11.5	11-64	27.6±10.8	14-67	0.019
Sex n (%)					
Female	38 (109)		52(129)		
Male	71(109)		77(129)		
RBC (10^6/ul)	5.11±0.51	3.80 -7.11	5.25±0.48	4.13-6.60	0.036
HGB (g/dl)	14.3±1.62	7.1-18	14.4±1.97	9.2-18.1	0.304
HCT (%)	41.5±4.21	25.9-50.3	42.2±4.63	30.1-51.9	0.259
MCV (fl)	81.3±4.02	64.4-90.8	80.2±5.9	61.5-95.4	0.111
MCH (pg)	27.7±2.41	17.7-32.4	27.4±2.91	17.9 -33.1	0.286
RDW (%)	13.4±1.56	11.7-20.9	13.6±1.87	11 -20.7	0.792
Serum FE (ug/dL)	76.6±34.7	14.1-190	87.6±43.8	15.1-290	0.081
TIBC (ug/dL)	347±44.02	265-473	340±45.8	234-664	0.208
Ferritin (ng/mL)	60.7±58.33	2.3-391	70.6±76.6	2.5-392	0.297
HbA2 (%)	4.16±0.45	2.05.2004	2.26±0.61	0.3-3.2	<=.001
HbA0 (%)	58.38±2.91	50.2-72.1	58.9±3.27	34.6-65.6	0.133
HbF (%)	0.08±0.28	0-2.1	0.04±0.16	0-1	0.173
Abnormal Hgb (%)	37.2±2.48	24.6-44.6	38.3±2.21	32.2-43.5	<=.001
Retention Time (min.)	1.02±0.01	1.01.2006	0.96±0.01	0.94-0.99	<=.001

Note. HGB: Hemoglobin; HCT: Hematocrit; MCV: Mean Corpuscular Volume; MCH: Mean corpuscular hemoglobin; RDW: Red blood cell distribution width; TIBC: Total iron-binding capacity ; Independent T test. Significant p < 0.05*

Discussion

In this study, hemogram results of patients diagnosed with Hb S carrier and Hb D Los Angeles carrier between 2015 and 2021 were compared with HPLC thalassemia screening tests haematologically and chromatographically, and their distinctive features were discussed.

The results of hematological studies of Hb S carriers and Hb D Los Angeles carriers showed that one of the most important distinguishing variables was HbA2 levels. The mean HbA2 level was 4.16% ± 0.45% in Hb S carriers, and 2.26% ± 0.61% in Hb D Los Angeles carriers, and this difference was statistically significant (p<0.001). In agreement with this study, Fonseca et al. compared sickle cell anemia and alpha thalassemia genotypes, it was reported that Hb A2 concentrations were affected in the presence of HbS or HbC, and their levels increased slightly [14]. In a study conducted by Suh et al. it was reported that HbA2 levels increase in the presence of HbS or HbC hemoglobin [15, 16]. Moreover in the study by Şokrani et al., in which they evaluated HbA2 levels in sickle cell anemia patients in the HPLC system, it was reported that HbA2 levels were high [16]. However, in the case of simultaneous eluting of HbS with HbA2 in HPLC systems, sometimes part of Hb S may overlap HbA2 and cause false height [15, 16] This may cause the sickle cell carrier to be mistakenly interpreted as Hb S/Beta. In this case, parental screening should be performed. Besides, it should be kept in mind that Hb A2, which is one of the hematological data, may cause false lows or false highs in conditions such as iron deficiency, megaloblastic anemia, alpha thalassemia, delta gene mutations, hypothyroidism, and paraproteinemia, and it does not have sufficient discrimination between Hb S and Hb D on its own [7,17].

One of the most important distinguishing variables among the variants in HPLC systems is the RT difference, which is formed depending on the elution pattern [18]. Although the Hb S retention time has a course around 1.01 and the Hb D retention time is around 0.96, it can rarely be detected at lower or higher RT than Hb S and HbD normally are [18].

A statistically significant difference was detected in serum RBC levels between Hb S carriers and Hb D Los Angeles carriers (p<0.001). A possible explanation for this situation is that the population we used includes different genders and age ranges, and there may be a significant difference in these parameters in Hb S and Hb D carriers due to the difference in the reference values of RBC parameters in the pediatric and geriatric groups [19].

In addition, abnormal hemoglobin levels showed statistically significant difference between Hb S carriers and Hb D Los Angeles carriers (p<0.001). While the minimum and maximum values of abnormal hemoglobin in Hb S and Hb D Los Angeles carriers rangebetween 24.6% and 44.6% in Hb S carriers, it is between 32.2% and 43% in Hb D carriers. According to our study, both variants can be in the percentage range of each other. For this reason, although there is a statistically significant difference on the basis of population in terms of abnormal hemoglobin, it may not have sufficient distinguishing features individually.

In a study by Anagnostopoulos et al., they examined the effect of the presence of Hb S and HbA2 in different devices and an increase in HbA2 levels was found [20].

There are a limited number of detailed studies on the hematological and molecular basis of Hb D. In the study by Higgins et al., it has been reported that HbDs cause falsely low HbA2 levels and HbS falsely cause high HbA2 levels [21]. This situation is generally compatible with other studies in the literature and our study results. The fact that HbD and HbS migrating together in alkali cellulose acetate electrophoresis leads to the mixing of the two variants and they cannot be separated from each other. Therefore, HbD and Hb S were tried to be distinguished in acidic citrate electrophoresis. In acidic citrate electrophoresis, false levels can be detected as a result of HbD migrating with HbA [7].

Although, today, with the ease of access to technology, Hb S and Hb D are now more easily separated in methods such as HPLC and capillary electrophoresis, which are widely used, it should be kept in mind that it can be confused due to gradient program errors, analytical errors, software differences, and common biochemical features [18]. Existing guidelines state that the Hb S variant should be confirmed with another technique with caution [13].

Conclusion

In conclusion, the Hb D-Los Angeles variant and the Hb S carrier profile have similar hemogram and electrophoresis, they can be distinguished from RT levels, HbA2 levels, and abnormal hemoglobin levels. Although RT and HbA2 levels seem to be the main distinguishing points, it should not be forgotten that they do not always have sufficient distinctiveness. In the presence of different pathophysiological variants, it may lead to misdiagnosis and affect subsequent generations [7, 17].

The most powerful side of this study is that, to the best of our

knowledge, this is the first study investigating the differences between Hb S and Hb D Los Angeles carriers using HPLC. Molecular diagnostic methods such as PCR and DNA chain analysis should be used for definitive diagnosis.

Limitations of the study

The main limitation of our study is the inability to compare phenotype, genotype, and hematological data due to the inaccessibility of patients' clinical status and genotype data. More comprehensive comparisons can be made by taking samples from different regions of Turkey.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest

None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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