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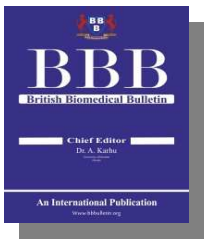

Original

A Long Term Screening of Iranian Populations with Thalassemia and Hemoglobinopathies

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ABSTRACT

Background: Thalassemias and hemoglobinopathies are genetic autosomal recessive disorders affecting hemoglobin molecules in different ways, qualitatively and quantitatively. The highest prevalence of thalassemia has been reported in the thalassemia belt and Mediterranean region countries and in Middle East including Iran. The present study evaluated the prevalence of α/ β-thalassemias and hemoglobinopathies in a large number of Iranian populations.

Methods: This five-year study was conducted on 3780 individuals. Initially complete blood cell count, HPLC, Hb electrophoresis and HbA_2measurement for thalassemia carrier identification. MCV≤80fL, MCH<27pg and Hb-A2>3.5% were standard diagnostics β-thalassemia diagnosis. In cases with low MCV and MCH indices (MCV≤80 fl, MCH<27 pg) and Hb-A2< 3.5% and normal Hb-electrophoresis, α-thalassemia was considered in the list of differential diagnosis. Patients with abnormal hemoglobin varieties in hemoglobin electrophoresis were candidate for more precise analysis with HPLC. In cases with low MCV for exclusion of iron deficiency serum ferritin was also measured.

Results: Our results revealed that 1932 (51.11%) had normal electrophoretic pattern, 781 (20.66%) had β-thalassemia trait and 487 (12.84%) had β-thalassemia major or intermedia, 328 (8.67%) had normal electrophoresis along with iron deficiency and 142 (3.75%) had normal Hb -electrophoresis and normal iron status but low MCV and MCH indices. We also identified 11 (0.29%) with Alpha thalassemia variants Hb-H disease/alpha trait and 22 (o.58%) with sickle trait and 18(o.47%) with sickle disease and 9 (0.23%) HbS-Thalassemia double heterozygote and 5 (0.13%) with E- trait and 32 (0.84%) with Hb-D variant and 1 (0.026%) with heterozygote Hb-C variant and 5 (0.13%) with Hb-D Iran and 1 (0.026%) with Hb-J trait and 1 (0.026%) HbS/D double heterozygote, and 1 (0.026%) with Hb-D/J double heterozygote

and 1 (0,026%) with Hb-constant spring/HB-H double heterozygote.
Conclusion: β-thalassemia is a common β-globin gene defect among Iranian and HPLC is a fast and reliable method in clinical laboratories especially in diagnosis of rare variant of hemoglobin and also as an important auxiliary tool for the diagnosis of thalassemia.

Introduction

Thalassemia syndromes and hemoglobin mutations are the most frequent genetic based disorders in human societies and various ethnic populations[1]. It has been recommended that all individuals from all ethnic groups except north European Caucasians be screened for variant hemoglobins, all ethnic groups for β-Thalassemia trait and selected ethnic group for α-thalassemia trait. And this could be a good reason why availability of a fast and reliable method for identifying these disorders in societies with high frequency plays a significant role.

It is especially important to validate the role of HPLC, as it is less labor intensive and has more rapid turn around time and better reproducibility compared to conventional Hb electrophoresis. This method could be considered as a valuable method for hemoglobinopathy and/or thalassemia carrier screening in couples from high-risk populations, identification of an abnormal neonatal hemoglobin and follow up of patients with thalassemia or hemoglobinopathy, post bone marrow transplant in order to analyses patterns of hemoglobin synthesis and in a way to document engraftment of donor hematopoiesis. Moreover, it can replace laborious procedures like estimation of fetal hemoglobin by chemical methods and HbA_2 quantization by column chromatography in clinical laboratories. A total of 340 globin gene mutations can cause α or β-thalassemia. These mutations down regulate or abolish the transcription of the corresponding globin genes or translation of globin MRNA. Among them 44 mutations such as Hb-E (β codon 26 GAG>AAG or Glu→Lys) Hb D-Iran (β codon 22 GAA > CAA or Glu→Gln), Hb lepore caused by δ-β fusion globins' chain, or Hb constant spring (α2 codon 142 TAA>CAA or Ter→Gln) can lead simultaneously to a variant hemoglobin and a Thalassemia phenotype because the normal globins is inefficiently made.

The measurement of HbA2 and HbF by HPLC is rapid, reproducible, and precise. It is as reliable as column chromatography for the measurement of HbA2 and radial immunodiffusion or alkaline denaturation for the measurement of HbF. HPLC may be an appropriate method for rapid screening in population surveys for beta thalassemia and HbE carriers which their detection are clinically important[2].

There are several indications for investigation of hemoglobinopathy and thalassemia[3]:

1. Clinical suspicion of thalassemia syndromes or sickle cell disease
2. Hemoglobinopathy and/or thalassemia carrier screening in couples from high-risk populations, for genetic counseling and reproductive decisions.
3. Confirmation and follow-up of an abnormal neonatal hemoglobin screening result.
4. Investigation of the family members of an individual known to have a hemoglobinopathy or thalassemia.
5. Preoperative screening for Hb S and other relevant mutations in high-risk populations.

6. Laboratory evidence of hemoglobinopathy and/or thalassemia such as,

a. Unexplained or familial polycythemia, hemolytic anemia, or cyanosis.
b. Positive Hb S solubility test.
c. Hemoglobin variant detected during Hb A1C determination.
d. Microcytosis, not due to iron deficiency.

The aim of the present study was to evaluate the role of cation exchange HPLC along with adjunctive tests as needed in the diagnosis of thalassaemias/haemoglobinopathies and to estimate the frequency of these disorders in the Iranian population.

Materials and methods

Patients, sample collection and early laboratory analysis

This was a study carried out in Gholhak clinical laboratory, over 5 year's duration from Aug 2006-Aug 2011. Consecutive 3780 blood samples sent to our laboratory for suspected thalassemia/ haemoglobinopathy work-up.

For laboratory analysis, 2-4 ml blood sample was collected in EDTA anticoagulant and was analyzed in automated cell counter (Mindray 5800; China) for complete blood counts and evaluation of hemoglobin concentration and Red Blood Cell Indices. Samples were stored at 4-8°C and were analyzed later in batches within 2-5 days.

Study protocol in diagnosis of hemoglobinopathy

We used HPLC analyzer [high performance liquid chromatography [Bio-Rad (D-10), USA] to identify normal and abnormal hemoglobins in patients with Hb-electrophoresis request. In this study by the use of HPLC-D-10- /Hb- A2/F Dual program. We had the ability to identify β-thalassemia trait carriers. This analyzer also had the ability to identify different types of Hb-variants like, S, C, D, E by the use of specific windows .In this study in order to validate our results and to follow the American college of pathologist accreditation that any non -S non A –Hb which is isolated with an HPLC method should be approved by another method (alkaline & acid electrophoresis by Sebia semi-automatic electrophoresis system)[4].

We used acetate cellulose alkaline electrophoresis at pH 8.2 to 8.6, although a standard in the past by the use of Sebia, semi-automated gel electrophoresis (HYDRASES, 2 scan, France) to identify common variants like Hb S and Hb C, but this technique does not have the ability to distinguish between HbS and $HbSD^{Punjab}$ and $G^{Philadelphia}$. Similarly, Hb A_2 is not readily separated from HbS, C, E, and O^{Arab}. Citrate agar electrophoresis at pH 6.0 to 6.2 provides better resolution for different hemoglobin variants. HbS/C, C^{Harlem}, E, and O^{Arab} which have different migrating mobility in this acidic medium. This could be used to distinguish HbS from HbS/D^{Punjab} or $G^{Philadelphia}$, but it cannot separate HbD^{Punjab} from $HbG^{Philadelphia}$, or HbA from HbA_2. This electrophoretic technique is still used in some laboratories as an aid to diagnosis of variant hemoglobins. We also used this technique for further distinction of variant hemoglobins[4].

The superiority of HPLC method to conventional electrophoresis techniques is that HbS and HbD have different retention times and are separated at different windows and can be distincted from HbG. In these cases we also examined the peripheral blood smear for sickle morphology and performed a slide sickeling test with NA- Metabisulfite to support our diagnosis. Similarly HbA2 can not be separated from HbC, HbE, HbO^{Arab} by conventional methods, in HPLC method, HbE also co-elutes with HbA2, but since HbO and HbC have different retention times from HbA2 in HPLC, in cases of HbA2>10% and according to the Bio-Rad

D-10 given report format pattern in these cases the isolated peak at Hb-A2 retention time might be suggestive of HbE variant[4,5].

HPLC method was also able to separately identify two hemoglobin variants of HbD family, HbD^{Iran} and HbD^{Punjab}. Both exhibited identical electrophoretic mobilities in conventional electrophoresis but eluted in A_2 and unknown window between S and C window (according to Bio-Rad instruction manual as D window) respectively on HPLC chromatogram. These situations are clinically important because HbD^{Punjab} produces a significant sickling disorder when present in a double heterozygous HbD/HbS form; whereas HbD^{Iran} is a clinically benign condition. The misdiagnosis of HbD^{Iran} as HbD^{Punjab} based solely on conventional hemoglobin electrophoresis or as HbE based solely on Hb- HPLC, where the subtle difference in Hb retention time is disregarded, may lead to incorrect genetic counseling[4,6].

Criteria for diagnosis of β-thalassemia and α-thalassemia

Elevated HbA_2 (>3.5%) with low MCV and MCH indices (MCV≤80 fl, MCH<27 pg) and normal hemoglobin pattern are the standard diagnostic markers for β-thalassemia. Hb F also was considered as a diagnostic factor in individuals suspected to β-thalassemia[6,7].

In this study cases with low MCV and MCH indices (MCV≤80 fl, MCH<27 pg) and Hb-A2< 3.5% and normal Hb-electrophoresis, α-thalassemia trait (αα/--) was considered in the list of differential diagnosis which in these cases HbH inclusion stain by brilliant cresyl blue to visualize Hb-H inclusions was performed. In these cases Iron deficiency anemia (IDA) was also considered as a differential diagnosis and these cases were evaluated for serum iron and serum ferritin levels[6,8].

Family HPLC screening also helped in some difficult cases especially in double heterozygous states. Role of family studies has been emphasized by other studies also.

Statistical analysis

Results were reported as mean ± standard deviation (SD) for quantitative variables and percentages for categorical variables. Statistical significance was based on two-sided design-based tests evaluated at the 0.05 level of significance. All the statistical analyses were performed by SPSS software.

Results

Study outcomes

Primary outcomes of this study were mean of MCV, MCH, MCHC and HbA2 in patients with β-thalassemia and α-thalassemia and the number of patients affected with these disorders. The number of individuals affected with different types of hemoglobinopathies was other another outcome of the study. Secondary outcomes were the number of patients with rare hemoglobinopathies and also determination of patients with different types of hemoglobinopathies. Determination of the number of patients with β-thalassemia major, thalassemia intermedia, homozygote of hemoglobinopathies and co-inheritance of two different defects were other outcome of this study.

From 3780 blood samples, 1932 (51.11%) had normal electrophoretic pattern, 781 (20.66%) had β-thalassemia trait and 487(12.84%) had β-thalassemia major or intermedia, 328 (8.67%) had normal electrophoresis along with iron deficiency (detected by low ferritin level and a repeated electrophoresis after sufficient iron therapy was recommended) and 142 (3.75%) had normal Hb electrophoresis and normal iron status but low MCV, MCH indices and normal range

of HbA2 (a presumptive diagnosis of an α-thalassemia trait was suggested and α/β chain ratio studies and DNA analysis was recommended). We also identified 11 (0.29%) individuals with alpha thalassemia variants (Hb-H disease/alpha trait) and 22 (0.58%) with sickle trait and 18 (0.47%) with sickle disease and 9 (0.23%) HbS/ thalassemia double heterozygote and 5 (0.13%) with HbE trait and 32 (0.84%) with HbD variant and 1(0.026%) with heterozygote HbC variant and 5(0.13%) with HbD^{Iran} and 1 (0.026%) with Hb-J trait and 1 (0.026%) HbS/D double heterozygote, and 1 (0.026%) with Hb-D/J double heterozygote and 1 (0,026%) with Hb-constant spring/HB-H double heterozygote. There were also 2 (0.053%) of cases with slight increase in HB-F due to pregnancy and 1 (0.026%) with moderate increase in HB-F in a known case of myeloid leukemia (Table 1).

Various normal and variant hemoglobin's encountered in our study according to retention times were as follows:

Hemoglobin variants with retention times <1.0 min

13 case were detected, which were confirmed by fast-moving band on electrophoresis and a positive Hb-H inclusion stain, 12 had HB-H disease/alpha trait and 1 had Hb-CS/H disease double heterozygote.

Hemoglobin variants with retention times in the F window

However, most of our patients with high Hb in the F window were mostly homozygous β-thalassemia patients or double heterozygous β thalassemia/haemoglobino-pathy patients confirmed by Hb-electro-phoresis and family studies. 3 cases revealed high Hb-F levels, 2 in the range of 1-10 per cent, were pregnant women and 1 of whom had AML with 30% HbF.

Hemoglobin variants with retention times in the p3 window

We found 2 cases of possible Hb-J which was suspected based on its retention time (1.58 min) these 2 were members of a family, mother with HbD^{Iran} heterozygote and father with Hb-J heterozygote and a child with HbD/J double heterozygote. We also confirmed our Hb-J wih Sebia capillary zone electrophoresis as a distinct band in J-window.

Hemoglobin variants with retention times in the A2 window

Three hemoglobin variants had elution peaks in the A_2 window including Hb A_2, HbE and HbD^{Iran}. The retention time for HbD^{Iran} (3.30 min) appeared to be different from those of HbE (3.437min). The values more than 40 percent in Hb A_2 window with a different retention time was suspected as HbD^{Iran} which was confirmed by alkaline electrophoresis, where a band in the SDG region was detected and also a negative sickle prep.

Hemoglobin variants with retention times in the between S and C window and unknown peak title

32 cases with this characteristics were detected which in conventional alkaline electrophoresis a peak had been isolated in SDG region and according to our Bio-Rad report format interpreted as HbD variant.

Hemoglobin variants with retention times in the S window

50 cases HbS disease/ trait HbS/D, with retention times of 4.27 min, were isolated in this window, 18 had Sickle disease, 22 had HbS trait and 1 had HbS/D double heterozygote.

Hemoglobin variants with retention times in the C window

two hemoglobin variant were detected in this window with (4.79 min.) retention time 1 with 33.8% HbC which was considered as HbC trait and 1 with 2.5% hemoglobin variant eluted at C window which could be a heterozygote Hb-constant spring (this patient had also About 11% Hb isolated at fast hemoglobin region (H-region in acetate cellulose) which was considered a double heterozygote Hemoglobin H/Constant Spring variant.

Discussion

In this study 20.66% had β-thalassemia trait which this high prevalence among Iranian population emphasizes the need for a fast and reliable method for premarital screening in our country we also had 12.84% β-thalassemia major or intermedia cases which has to be investigated that why despite of premarital investigations thalassemia major and intermediate infants are still born and it seems that there should be more education in high schools about these congenital anemias and a cultural education that before making any decisions about marriage first some screening tests has to be taken[5-7]. We also identified (0.29%) individuals with Alpha thalassemia variants Hb-H disease/alpha trait and (0.58%) with sickle trait and (0.47%) with sickle disease and (0.23%) HbS/Thalassemia double heterozygote and (0.13%) with E- trait and (0.84%) with Hb-D variant and (0.026%) with heterozygote HbC variant and (0.13%) with HbD^{Iran} and (0.026%) with Hb-J trait and (0.026%) HbS/D double heterozygote, and (0.026%) with Hb-D/J double heterozygote and (0,026%) with Hb-constant spring/HB-H double heterozygote which shows the necessity for a reliable method for screening these cases which might have a normocytic normochromic anemia and might be missed in heterozygote states if our screening methods are not reliable[8-10].

There are a variety of approaches worldwide to screen for α- and β-thalassemia that are based on the clinical presentation of these disorders. A relatively common approach which was also had been chosen in our study consisted of a complete blood count to assess the mean cell volume and the mean cell hemoglobin. The finding of abnormal MCV (i.e≥80 fL) in combination with a normal MCH (i.e., ≥27 pg) would rule out most cases of thalassemia and would require no additional thalassemia testing [5, 6]. For individuals with MCV<80 fL or MCH<27 pg, the next step is hemoglobin electrophoresis or HPLC, quantitation of HbA2 and HbF, and a blood smear stained for H inclusions. This approach However, may fail to detect carriers of hemoglobinopathies such as HbS, HbC, or HbD, because hemoglobinopathy heterozygotes may have a normal CBC (normal MCV and normal MCH)[7,11]. Given that homozygosis and compound heterozygosis for a hemoglobinopathy/thalassemia can produce very significant morbidity and early mortality. Hemoglobinopathy screening may be as important as screening for thalassemias. Detection of hemoglobinopathy carriers cannot be reliably performed by CBC alone and requires hemoglobin HPLC or electrophoresis. An MCV ≥80 fL and an MCH ≥27 pg, with a normal electrophoresis or HPLC, requires no further testing and we recommended in our comments that other causes of anemia has to be investigated. Individuals with an MCV < 80 fL or MCH < 27 pg can have α- and/or β-thalassemia and/or iron deficiency anemia. In general, β-thalassemia trait can be reliably diagnosed by hemoglobin electrophoresis or HPLC, with HbA2 and HbF quantization. Patients with β-thalassemia trait have an elevated HbA2, i.e., > 3.5% [4, 6]. In patients with a low MCV, but with a normal Hb electrophoresis/HPLC and HbA2 and HbF quantization, the

differential diagnosis includes iron deficiency anemia and α-thalassemia trait[5]. A serum ferritin (to exclude iron deficiency anemia) and a smear to screen for the H- inclusions in α-thalassemia trait are therefore required. H -inclusion stain is not 100% sensitive and therefore the absence of H -inclusions does not completely exclude α-thalassemia carrier status in an ethnically at-risk patient. In this group we recommended molecular studies for α-thalassemia mutations (e.g., multiplex PCR). HPLC method will also allow identification of Hb variants, such as HbS, C, D, and E. A sickle cell preparation (such as a slide or tube sickling test) is not helpful in identifying other types of β-globin variants besides HbS but since our HPLC system had a C and D window and with this system E-hemoglobin also coeluted with Hb-A2, in cases with Hb A2>10% a coelution with HbE variant was considered as a presumptive diagnosis[9,10].

Since HPLC method has high ability to isolate different Hb variants based on their retention time and to identify β-thalassemia carriers much faster than the conventional methods (acid and alkaline electrophoresis), we recommend it as a fast and reliable method in clinical laboratories especially in premarital, and neonatal screening laboratories. It is diagnostic in most cases and only a few require other modalities for validation. Retention time and percentage of variant hemoglobin can provide important clues in differentiating variant hemoglobin's eluting in the same window[4,6].

Conclusion

The data summarized here confirm that screening for hemoglobin disorders should be an intrinsic part of health care in most countries, as recommended by the WHO[3].

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Table 1. Laboratory findings about distribution of normal and abnormal haemoglobin variants

Hb Pattern	No. of cases	Percentage	Hb (g/dl)
Normal	1932	(51.11%)	9.9
β-thalassemia trait	781	(20.66%)	11.1
β-thalassemia major or intermediate	487	(12.84%)	7.2
Normal electrophoresis along with iron deficiency	328	(8.67%)	8.9
Normal Hb -electrophoresis and normal iron status but low MCV and MCH	142	(3.75%)	12
Hb-D variant	32	(0.84%)	9.9
Sickle trait	22	(0.58%)	6.2
Sickle disease	18	(0.47%)	7.5
Alpha thalassemia variants Hb-H disease/alpha trait	12	(0.29%)	9.1
S-Thal double heterozygote	9	(0.23%)	6.6
HbE- trait	5	(0.13%)	12
Hb-D Iran	5	(0.13%)	7.9
Slight increase in HB-F due to pregnancy	2	(0.053%)	11
Heterozygote Hb-C variant	1	(0.026%)	11
Hb-J trait	1	(0.026%)	10
Hb-D/J double heterozygote	1	(0.026%)	
Hb-S/D double heterozygote	1	(0.026%)	8.7
Hb-constant spring/ Hb-H double heterozygote	1	(0.026%)	9
Hb-F in AML	1	(0.026%)	12