PREVALENCE OF HEMOGLOBINOPATHIES AND HEMOGLOBIN VARIANTS

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ABSTRACT

Hemoglobinopathies are group of inherited disorders which can broadly be classified into qualitative and quantitative defects. Diagnosis of hemoglobinopathies relies upon various methods involving clinical and family history, complete blood counts (CBC), red cell indices, HbA2, HbF estimation, sickling test, and Hb electrophoresis, capillary electrophoresis and high performance liquid chromatography. The aim of our study was to find out the various hemoglobinopathies in patients with anemia. This was a cross-sectional observational study performed at Kathmandu Pathlab and Diagnostic centre Pvt. Ltd. All the suspected case of hemoglobinopathies and sent for HPLC/Electrophoresis; from January 2016 to July 2016; were included in the study. Reports and chromatograms generated were studied and interpreted by observing HbA2 and F concentration for β thalassemia and retention time and area percentage of other peaks and windows for structural variants. Out of 163 cases undergoing HPLC; 86 (52.7%) didn't have hemoglobinopathies and 77 (47.3%) with female to male ratio of 1.48:1. Most common hemoglobinopathies in this study was sickle- β thalassemia (14.1%), followed by sickle cell disease (13.5%); which included both sickle cell trait and sickle cell anemia; and β thalassemia (12.9%). Hereditary Persistence of Fetal Hemoglobin was more prevalent in nonethnic population than ethnic population (11.8% vs. 3.1%). Though hemoglobinopathies remains prevalent in ethnic population, due to internal migration of population, hemoglobinopathies should be kept in mind while managing anemia. In this study most common hemoglobinopathies was sickle-beta thalassemia followed by sickle cell disease and beta thalassemia.

KEYWORDS

HbE, HPFH, HPLC, sickle cell, thalassemia

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INTRODUCTION

Hemoglobinopathies are group of inherited disorders which can broadly be classified into qualitative and quantitative defects. Qualitative defects include Sickle cell anemia whereas quantitative defect includes Thalassemia. Sickle cell anemia is characterised by abnormality in the structure of haemoglobin in particular substitution of adenine in sixth codon of β gene (GAG-GTG), thereby encoding valine instead of glutamic acid in sixth position of β chain.[1] In thalassemia there is reduced production of one or more globin chains. Thalassemia is generally classified into two broad categories: α-thalassemia and β-thalassemia usually caused by deletions of one or all four alleles of α- genes and point mutation β gene respectively. This results in reduction or absence in globin chain synthesis.[2]

Sickle cell disease is one of the most common genetic pathologies in the world. It comprises sickle cell anaemia and other compound heterozygous state such as haemoglobin SC disease, Sβ-thalassemia, and SD-Punjab. About 5% of the world's populations are carriers of genes responsible for hemoglobinopathies and about 300,000 children are born annually with haemoglobin disorders.[3]

It is characterized by homozygous hemoglobin S (Hb S) or Hb S associated to other Hb variants.[4] There is great clinical variation in the clinical manifestations between sickle cell disease patients. Several factors are associated with the different presentations. Some determinants, such as genetic, clinical and laboratory factors are already well established while others, such as psycho-social and nutritional factors, have been less well studied.[5-8]

Of the genetic factors, the importance of the phenotype of the hemoglobinopathy is well characterized in that individuals doubly heterozygous for sickle cell anemia and those with Hb S/β0-thalassemia have a more severe clinical profile. On the other hand, carriers of Hb SC together with Hb S/β+-thalassemia have a better outcome, which makes the correct diagnosis of these syndromes an issue of great importance for a better understanding and adequate clinical and therapeutic management of patients.[7-9]

Other hemoglobin variants frequently detected are hemoglobin C (Hb C) and hemoglobin E (Hb E). These variants have a lysine residue instead of a glutamic acid at the 6th (Hb C) and 26th (Hb E) positions of the β -globin chain.

Diagnosis of hemoglobinopathies relies upon various methods involving clinical and family history, complete blood counts (CBC), red cell indices, HbA2 , HbF estimation, sickling test, and Hb electrophoresis, capillary electrophoresis and high performance liquid chromatography. The aim of our study was to find out the various hemoglobinopathies in patients with anemia.

MATERIALS AND METHODS

This is a cross-sectional observational study performed at Kathmandu Pathlab and Diagnostic centre Pvt. Ltd. All the suspected case of hemoglobinopathies and sent for HPLC/ Electrophoresis; from January 2016 to July 2016; were included in the study.

Blood samples were collected in ethylene diamine tetrachloride acetate (EDTA) vials and analyzed with Sysmex, United States of America (USA) automated cell counter for complete blood counts.Complete blood count, Red cell indices, peripheral blood smear examination were performed from EDTA sample.

HbA2, HbF, and other haemoglobin variants were studied by HPLC method used for chromatographic separation of human haemoglobin (BioRad). Five millilitres (5mL) of whole blood was collected in a vacuum collection tube containing EDTA which can be stored at 2–8^{0}C for maximum 7 days if processing is delayed. No preparation was required. HbA2/F calibrators and normal and abnormal controls were analysed routinely.

Reports and chromatograms generated were studied and interpreted by observing HbA2 and F concentration for β thalassemia and retention time and area percentage of other peaks and windows for structural variants. Each chromatogram shows peaks of Hb A0, A2, and HbF along with C window, D window, S window, and two minor peaks, P2 and P3. Several haemoglobin variants elute same window; they were provisionally diagnosed by retention time and area percentage keeping in mind the ethnicity of the patients and confirmed by capillary electrophoresis.

Relevant data were collected which included demographic data, haematological parameters and peripheral blood smears findings were correlated with interpretations of HPLC. All the findings were analysed using SPSS Vs.21.

RESULTS

The mean age of the patients coming for HPLC testing was 20.45 ±11.98 years with slight female predominance having female to male of 1.36:1. Ninety eight percent of the patients were from Dhangadi district. Aboriginal population to nonethnic population ratio in this study was 1.43:1. Mean age of the patients with hemoglobinopathies was 19.1±11.3 years. Out of 163 cases undergoing HPLC; 86 (52.7%) didn't have hemoglobinopathies and 77 (47.3%) with female to male ratio of 1.48:1, had hemoglobinopathies.

Various haematological parameters were studied which included RBC count, Hemoglobin (Hb), Hematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Red cell distribution width (RDW) and peripheral blood

smears. Mean of these parameters are shown in Table 1: Mean of various haematological parameters among 77 patients with hemoglobinopathies are tabulated in Table 2. No significant differences were observed among Mean MCV and MCH in different hemoglobinopathies. However, RDW was increased in patients with B Thalassemia major, minor or in compound condition of HbS and B Thalassemia (P<0.001). Red cell distribution width was even more increased in HbH disease, HbE and HPFH. On peripheral blood smears 42.3 % (n=69) were of normocytic normochromic, 39.9% (n=65) were microcytic hypochromic and 17.8% (n=29) were of macrocytic normochromic. (Table 3) Out of hemoglobinopathies, 58% of patients associated with defect in B globin chain had microcytic hypochromic anemia compared to 43% in patient with Sickle cell disease alone.

Table 1: Mean hematolgical parameters in patients having HPLC tests.

	RBC million/cmm	Hb gm/ dl	PCV %	MCV fL	MCH pg	RDW CV
Mean	3.3987	7.9425	28.429	85.365	25.490	22.4576
Std. Deviation	1.51	3.44	12.00	16.20	14.71	7.05

Table 2: Mean values of various haematological parameters in hemoglobinopathies

Interpretation		RBC mln/cmm	Hb gm/dl	PCV %	MCV fL	MCH pg	RDW CV
Homozygous Sickle cell disease	Mean	3.23	8.05	26.80	83.65	39.69	18.98
	SD	1.41	2.59	8.18	11.51	51.86	2.26
Heterozygous Sickle Cell disease	Mean	4.14	9.21	33.75	81.20	22.76	19.07
	SD	1.63	4.02	14.56	4.60	1.62	4.20
Sickle cell disease with β thalassemia	Mean	3.53	8.01	27.96	80.39	23.16	22.16
	SD	1.39	2.91	10.11	8.95	2.33	6.19
β thalassemia major	Mean	1.94	3.60	13.00	73.80	20.65	38.30
	SD	1.88	3.11	11.17	13.85	4.31	5.79
β thalassemia minor	Mean	3.89	8.06	29.83	81.55	22.67	21.88
	SD	1.73	3.56	10.97	16.69	3.92	5.86
HPFH	Mean	2.22	5.22	18.30	89.18	25.82	25.18
	SD	1.19	2.04	6.94	15.87	5.21	5.25
HbE disease	Mean	5.06	9.90	35.80	70.80	19.60	21.10
Compound HbE with β thalassemia minor	Mean	4.25	9.20	31.50	74.10	21.60	29.50
HbH disease	Mean	5.34	8.10	30.50	57.10	15.20	36.70

Table 3: Different haemoglobin patterns observed in various hemoglobinopathies

INTERPRETATION		HbA	HbA2	HbF	Peak3	Sickle	Other
Normal	Mean	91.41	2.28	1.28	5.04	0.00	1.62
	SD	3.08	0.73	0.71	1.95	0.00	2.17
Homozygous Sickle cell disease	Mean	16.65	2.55	20.01	0.18	64.88	1.95
	SD	12.84	1.17	7.14	.478	13.82	2.05
Heterozygous Sickle Cell disease	Mean	59.58	3.00	6.91	3.23	29.60	1.62
	SD	11.71	1.03	9.16	2.09	6.28	1.47
Sickle cell disease with β thalassemia	Mean	35.94	4.93	15.86	1.43	45.21	0.95
	SD	25.87	0.92	11.53	1.78	21.18	0.52
β thalassemia major	Mean	9.65	2.00	93.70	0.00	0.00	2.20
	SD	12.09	2.828	4.38	0.00	0.00	2.96
β thalassemia minor	Mean	88.43	5.12	2.31	5.68	0.00	1.09
	SD	2.88	1.21	1.63	2.99	0.00	1.58
HbH disease	Mean	79.70	1.00	2.20	2.20	0.00	0.50
HPFH	Mean	81.50	1.93	14.85	4.28	0.00	1.66
	SD	7.54	1.18	9.97	1.27	0.00	1.37
HbE with β thalassemia	Mean	13.40	75.10	30.10	4.60	0.00	1.10
HbE disease	Mean	5.80	2.50	4.00	0.30	0.00	7.60

Different types of Hb are identified in HPLC namely HbA, HbA2, Peak 3, Sickle and Other. Interpretation of results of HPLC was done on the basis of retention time, percentage of Hb, and peak characteristics.

Certain haemoglobin variants are seemed to be prevalent depending on hemoglobinopathies. (Table 4) One (0.6%) case of HbH was detected, which was suspected on the basis of a significant peak that

appeared in each case during the first minute of elution. Confirmatory tests were performed, which included Hb electrophoresis. No Hb variant was observed in the p1 window (retention time: 0·63-0·85 min). High Hb level in the F window having retention time 0·98-1·2 min was detected in three different cases, namely, β thalassemia major (n=2; 1.2%), Eβ thalassemia (n=1; 0.6%), sickle-β thalassemia (n=23; 14.1%), sickle-cell disease (n=22; 13.5%), and hereditary persistence of fetal hemoglobin (8; 4.9%). A_0 window has a retention time between 1.9 min and 3.1 min. Apart from HbA, no other Hb variant was found to elute in this window. HbA2 and HbE were found to elute in A2 window (retention time: 3.3-3.9 min). HbE disease was detected in 0.6% case and Eβ thalassemia in 0.6% cases. It was confirmed by capillary electrophoresis. HbS was found to elute in the S window (retention time: 4.3-4.7 min). In this study, sickle-cell trait was found in 10 (6.1%) cases and sickle-cell disease was noted in 12 (7.4%) patients. Detail varieties of diagnosis are tabulated in Table 5. Among aboriginal community 14.8% were of defect in sickle haemoglobin, 14.8% with heterozygous HbS with β-Thalassemia and 10.2 % with βThalassemia. Among nonethnic community only 8.8% had defect in Sickle haemoglobin, 11.8% with heterozygous HbS with β-Thalassemia and 17.6% with βThalassemia. In male compound sickle-β thalassemia was seen in 17.4% of all the male patients included in this study followed by Sickle cell disease (17.3%). In female β-Thalassemia comprises 17.0% followed by sickle- β thalassemia (11.7%) and sickle cell disease (10.6%).

Table 4: Prevalence of hemoglobinopathies among patients undergoing HPLC

Diagnosis	Frequency (%)
Normal	86 (52.8)
Homozygous sickle cell disease	12 (7.4)
Heterozygous sickle cell disease	10 (6.1)
Sickle- β thalassemia	23 (14.1)
β thalassemia minor	19 (11.7)
HPFH	8 (4.9)
β thalassemia major	2 (1.2)
HBH disease	1 (0.6)
HbE with β thalassemia minor	1 (0.6)
HbE disease	1 (0.6)
Total	163 (100)

Table 5: Morphological types of anemia in various hemoglobinopathies

Normal	Interpretation										Total
	Homozygous sickle	Heterozygous sickle	Sickle- β thalassemia	β Thalassemia	HBH	HPFH	HbE with β thalassemia	HbE	β Thalassemia major		
Microcytic hypochromic	26	4	5	14	10	1	2	1	1	1	65
Normocytic Normochromic	39	7	5	8	5	0	4	0	0	1	69
Macrocytic Normochromic	21	1	0	1	4	0	2	0	0	0	29
Total	86	12	10	23	19	1	8	1	1	2	163

DISCUSSION

Haemoglobin comprises four globin chains: fetal haemoglobin (Hb F) has two α and two γ chains (α2γ2) and adult haemoglobin (Hb A) has two α and two β chains (α2β2). Genes in the α-globin and β-globin gene clusters (on chromosomes 16 and 11) control globin-chain production. Due to spontaneous mutation, haemoglobin gene variants are present at low prevalence (carriers 1–1.5/1000) in all sizeable populations.[10-11] They fall into two broad groups – structural variants that change the amino acid sequence and produce an unusual haemoglobin, and thalassemia that lower or abolish production of globin chains.[12,13] Most haemoglobin gene variants are rare and many are harmless, but some are common because carriers are less likely than others to die from falciparum malaria. The most common such variant, α plus (α+) thalassemia, is usually harmless. However, people who inherit combinations of haemoglobins S, C, E, D Punjab, β thalassemia, or α zero (α0) thalassemia may have a serious haemoglobin disorder. In populations in which malaria is (or was) endemic, 3 to 40% of individuals carry one of these significant variants, and the prevalence of haemoglobin disorders ranges from 0.3 to 25 per 1000 live births.[14]

Carriers are easily detected by routine haematological methods and can be forewarned of their reproductive risk. Carriers of structural variants have 30–50% of the variant haemoglobin in their red cells: thalassemia carriers have small red blood cells and sometimes mild anaemia, and β thalassemia carriers also have over 3.5% of Hb A2. The resemblance between thalassemia and iron deficiency can confuse the diagnosis of either disorder.[15]

In our present cross-sectional observational study, the prevalence of Hb disorders was found to be 47.2%, which was higher than other studies conducted in a nearby country India, where the prevalence rate was 12.17% and 25%.[16-18] Since our study included several aboriginal habitants suffering from anemia from anemia; prevalence of hemoglobinopathy may have increased.

Most common hemoglobinopathies in this study was sickle- β thalassemia (14.1%), followed by sickle cell

disease (13.5%); which included both sickle cell trait and sickle cell anemia; and β thalassemia (12.9%). Large scale studies regarding hemoglobinopathies has not been conducted in Nepal. In a study done by Shrestha A *et al* 37.1 % patient had increase band of HbA2 and HbS.[10] In studies done in India only few cases of sickle- β thalassemia have been encountered.[16,19] In contrast to our study; very high percentage (65.71%) of sickle cell disease was observed in a study.[10] This may be due to different sites of residence in Nepal and their ethnicity. Similarly β thalassemia was among the third most common case in this study. In India β thalassemia remains the most common type of hemoglobinopathies but with similar incidence (12.5%).[18]

In this study hemoglobinopathies were observed in (n=59/128; 46.1% of aboriginal community which was even more in nonethnic community (n=18/34; 52.9%). This may be due to more numbers of ethnic community (Chaudhary; 78%) were included for study suspecting hemoglobinopathies in comparison to nonethnic community.

Origin of Hb S and its mutation has been seen in several locations within Africa and Asia. Sickle Hb containing red cells inhibits proliferation of plasmodium falciparum, and is more likely to become deformed and removed from the circulation. Recently, due to movement of populations dissemination of sickle mutation in different areas of the world took place.[20] According to the study, Sickle hemoglobin seen majority in Tharus from malarial endemic region and minority of patients are of different ethnic groups of Nepal. Though malarial hypothesis explains sickle hemoglobin in Tharus, it's not answerable in other ethnic group.

Hereditary Persistence of Fetal Hemoglobin was more prevalent in nonethic population than ethnic population (11.8% vs. 3.1%). In a study conducted by Mondal SK *et al* very low prevalence rate of HPFH (0.18%) was observed in West Bengal.[16] Other studies also had low prevalence rate of HPFH.[21,22]

RBC indices are valuable in the provisional diagnosis of hemoglobinopathies. In qualitative defect MCV and MCH remains in normal or low normal range. However, in quantitative defect MCV and MCH remain in lower side of the reference range. In this study though microcytic hypochromic anemia was more prevalent in anemic individuals with hemoglobinopathies, no significant differences were observed among Mean MCV and MCH in different hemoglobinopathies. Mean corpuscular volume was reduced in thalassemia patients in comparison to sickle cell anemia. Similar findings were observed in another study.[16] However, RDW was increased in patients with B Thalassemia major, minor or in compound condition of HbS and β thalassemia. RDW indicates wide range of Red blood cells' corpuscular volume, which is more indicative of nutritional deficiency anemia than hemoglobinopathies. The significant increase in RDW in this study indicates coexisting iron deficiency anemia or megaloblastic anemia or sickle cell anemia.[23]

On peripheral blood smear examination, 50.06% of patients with hemoglobinopathies had microcytic anemia followed by 30.9% of patients had normocytic normochromic anemia and 19.04% had macrocytic anemia. Among other anemic patients included in the study and not detected hemogloinopathies had 30% microcytic hypochromic anemia followed by 45.3% with normocytic normochromic anemia and 30.45% with macrocytic normochromic anemia. It is well established that in thalassemia, peripheral blood smear reveals microcytic hypochromic anemia and borderline normocytic normochromic anemia and Macrocytic normochromic anemia in HPFH, βthalassemia major. However, coexistent nutritional deficiency anemia may mask the picture.

Despite being a sensitive, specific, and accurate technique for the identification and quantification of different Hb fractions, it should be kept in mind that HPLC has some limitation. It is unable to detect α thalassemia and normal HbA2 β thalassemia. Hb variants that elute with the same retention time also cannot be separately identified by HPLC and during interpretation of chromatograms, nutritional anemias must always be taken into account. A low level of HbA2 may be induced by iron deficiency, thus masking β thalassemia trait. Similarly, cobalamin or folate deficiency may raise HbA2 level, leading to a false diagnosis of thalassemia trait and whenever necessary, HPLC must be followed by molecular studies, such as polymerase chain reaction (PCR), amplification refractory mutation system (ARMS), and other similar tests to determine specific mutations responsible for the Hb disorder.[16,25]

In conclusion, though hemoglobinopathies remain prevalent in ethnic population, due to internal migration of population, hemoglobinopathies should be kept in mind while managing anemia. In this study most common hemoglobinopathy was sickle-Beta thalassemia followed by sickle cell disease and beta thalassemia.

HPLC method is sensitive and accurate technique for the identification and quantification of Hb variants. However, it coelutes with different Hb fraction, hence one should be cautious while making diagnosis and it must be followed by molecular studies.

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