Original Article

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Morphological Patterns of Anaemia and Prevalence of Haemoglobinopathy in State of Haryana: Study from a Tertiary Hospital

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ABSTRACT

Background: Hemoglobinopathies including thalassemias are important prevalent group of erythrocytic genetic disorders found in various parts of the world and are important cause of morbidity and mortality.

Methods: Samples were run on 5 part differential cell counter and after analyzing various RBC parameters and peripheral smear, the appropriate sample on biorad HPLC for hemoglobinopathy and mutation study was done. Statistical analysis performed. Study population included two groups- anemic pregnant women and students.

Result: Out of 361 cases; 27 were positive for haemoglobinopathies giving a prevalence of 7.5 % in anemic mothers. Positive cases included 22 cases of β-thalassemia trait, 2 HbS trait, 1 case of HbD trait, 1 case of HbE trait and 1 case of HbE homozygous. Out of a total of 630 cases, 38 were found positive for various haemoglobinopathies including 36 cases of β-thalassemia trait, 1 case of thalassemia intermedia and 1 case of HbLepore. DNA analysis was done for 10 cases in which the common mutation found was IVSI-5 followed by codon 41/42 and codon 8/9

Conclusion: Proper screening studies should be done to diagnose the cases and reduce the prevalence of thalassemia major by informing and educating such cases.

Keywords: *Hemoglobinopathy, Haryana, Prevalence, Thalassemia Minor*

Introduction

Hemoglobinopathies including thalassemias are important group of erythrocytic genetic disorders and are prevalent in various parts of the world including India constituting important causes of morbidity and mortality. Thalassemia syndromes are autosomal recessively inherited group of disorders of haemoglobin synthesis characterized by absence or reduced formation of one or more globin chains. [1]

Clinically, the thalassemias are classified into major, intermediate and minor forms according to their presentation and severity. Thalassemia major is the most severe disorder in which patient needs regular blood transfusion. Patients with thalassemia intermedia clinically presents with anaemia and splenomegaly and transfusion is not required on regular basis. Thalassemia minor is the symptomless carrier state.[2] Interactions of beta thalassemia with other hemoglobin variants like HbD, HbS and HbE also produce thalassemic manifestations and constitute major health problem in India.[3]

The α-thalassaemias presents as four clinical subsets which gives reflection of the extent of impairment in α-globin chain production: silent carrier, α-thalassemia trait, Hb H disease and hydrops fetalis.[4]

Mainstay of treatment include regular blood transfusion alongwith iron chelation therapy. The only permanent treatment of thalassemia is bone marrow stem cell transplantation which is not possible in every case due to donor scarcity or some monitory status as the disease put large burden on patients. Prevention is the cost effective strategy to reduce the burden of disease and this may be attained by population screening, genetic counseling and prenatal diagnosis. In developing countries like India with limited resources require prevention of the disease by various means to decrease the financial burden and disease morbidity.

High mutation rate alongwith presence of genetic modifiers make the disease more complicated. Beta thalassemia are known to be caused by more than 300 mutations. So, alongwith screening of carriers the knowledge of different mutations are important in making diagnosis. The

compound heterozygous disorders and unusual variants like HbJ are all clinically significant revealing varying degree of severity. The detection of heterozygous carrier parents of these inherited disorders can prevent the homozygous state in the offspring.

Basic hematological methods for carrier detection includes determination various red cell indices determination and haemoglobin pattern analysis.[5] High Performance Liquid Chromatography gives an accurate quantification of HbA2 and HbF and detects large majority of Hb variants.[6] This technique is a highly sensitive and specific method for diagnosis of hemoglobinopathy and offers the distinct advantage over classic haemoglobin electrophoresis by quantitating abnormal haemoglobins. Confimatory identification usually requires DNA analysis or aminoacid sequencing.[7]

The present study is planned as a pilot project to screen antenatal mothers, adolescents and young adults for the pattern of anaemia, prevalence of various haemoglobinopathies and conduct extended family screening of patients with carrier or disease state.

Materials and Methods

The present study was conducted in department of Pathology, Pt. Bhagwat Dayal Sharma, University of Health Sciences, Rohtak. The study included antenatal anaemic mothers having haemoglobin(Hb) less than 10 gm% attending the department of Gynaecology and willing to enroll for the study were randomly selected and subjected to screening procedure. Second group included students enrolling to various courses being run in constituent colleges of Pt.B.D.Sharma University of Health Sciences. A detailed haematological workup included haemogram with Hb levels, RBC Indices including MCV, MCH, MCHC, RBC Count and RDW-CV were performed on 5- part differential automated blood cell counter. On the basis of any one or combination of the following screening criteria : Hb<10 gm%, MCV ≤ 75 fl, MCH ≤ 27 pg, RBC Count ≥ 4.5 million, microcytic hypochromic picture further screening was done.

Peripheral blood smear was prepared and stained with Leishman stain according to standard technique and examined for red cell morphology, anisopoikilocytosis, presence of target cells, tear drop cells, fragmented red cells, nucleated cells, haemoglobin distribution and presence of any inclusion. Reticulocyte preparation was made and reticulocyte count was carried out using supravital staining with methylene blue according to the standard technique.[8]

Anaemia was classified as normocytic, macrocytic, microcytic and dimorphic according to the morphology of red blood cells and comparing the size of red blood cells with the diameter of small lymphocyte nucleus.

The samples were subjected to High Performance Liquid Chromatography (HPLC) Biorad Varaint-II system for variant analysis of the samples for various haemoglobinopathies. Samples were stored between 2-8^{0}C maximum for a period of 7 days and subjected to HPLC analysis in batches. The instrument was calibrated with a haemoglobin A2/F haemolysate supplied by the manufacturer. Samples were run together with two levels of A2/F controls, also supplied by the manufacturer, in each run.

Data Analysis: Data was entered in Microsoft excel mastersheet and analysed using SPSSv20 software. Descriptive statistics (mean,standard deviation, range, percentages) was applied wherever appropriate. Statistical analysis performed using Pearson correlation test, independent t test, ANOVA test and ROC curve.

Mutation Study: In ten cases DNA analysis was done for mutations with ARMS primer in a single PCR assay. DNA was extracted from whole blood using NaCI/ Ethanol precipitation method and tested by agarose gel electrophoresis.[10] Amplification refractory mutation system polymerase chain reaction (ARMS-PCR) is a very sensitive method used to detect the known point mutations for genetic testing of β-thalassemia patients.[11] Multiplex ARMS-PCR was used for detection of IVS1-5 (G→C); Codon 8/9 (+G); IVS1-1 (G→T); and Codon 41/42 (-TCTT) mutation. PCR products were run on ethidium bromide containing 2.5% agarose gel for 1hour on 100 Volt. Then gel picture were taken on Gel Doc system.

Result

We analysed the two target groups separately(table 1). Antenatal group included total 361 cases, most of them were in the second trimester(60.1%). Out of 361 cases; 27 were positive for various haemoglobinopathies giving a prevalence of 7.5 % in anemic mothers. The positive cases included 22 cases of β-thalassemia trait, 2 cases of HbS trait, 1 case of HbD trait, 1 case of HbE trait and 1 case of HbE homozygous (table 2).

Various morphological patterns of peripheral smear were observed which included 246 (68.1%) cases of microcytic hypochromic anaemia, 27 (7.5%) cases of macrocytic anaemia, 9 (2.5%) cases of dimorphic picture and 79 (21.9%) cases had normocytic normochromic peripheral smear. All the positive cases had microcytic hypochromic picture on peripheral smear.

Student group included total 630 subjects 38 were found positive for various haemoglobinopathies including

36 cases of β-thalassemia trait, 1 case of thalassemia intermedia and 1 case of Hb Lepore. Prevalence of overall haemoglobinopathies in student cases came out to be 6.0 %.

Fourteen family members of the positive subjects also came forward for screening. They included 6 males (42.8%) and 8 females (57.2%). All the cases were found positive for haemoglobinopathies including 11 cases of β-thalassemia trait, 1 case of thalassemia intermedia, 1 case of HPFH and 1 case of HbE homozygous. Microcytic hypochromic peripheral smear was observed in all the cases.

Comparison of RBC parameters between cases of thalassemia syndrome Vs others (table 3) was also done and P value was found statistically significant in MCV, MCH, RBC count and retic count.

Various morphological patterns of peripheral smear were observed which included 148 (38.1%) cases of microcytic hypochromic anaemia, 24 (5.0%) cases of macrocytic anaemia, 3 (0.6%) cases of microcytic normochromic picture and 300 (63.1%) cases had normocytic normochromic peripheral smear. All the positive cases of haemoglobinopathies had microcytic hypochromic picture on peripheral smear.

Out of total 79 positive cases DNA analysis was done in 10 cases in which most common mutation found was IVSI-5 followed by codon 41/42 and codon 8/9. But the number of cases were too small to conclude anything about mutation analysis. (photograph 1).

All cases belonged to different ethnic groups including Jat, Punjabi, Baniya, Backward Caste group (yadav, khatri, aheer, saini, gujjar, nai, sunar, bairagi, kumhar, chippi, maniyar) and Schedule Caste group (chamar, dhanak, balmiki, khatik, bawaria) and 90 cases did not disclosed their caste.

Limitations of the study: Population size was small and distribution of sexes was obvious as antenatal people are only female. Sex distribution can be doneonly for student group. Family members were not willing to enroll in the screening procedure so extended family study was not possible in large number of cases. Size for mutation study was also very limited so it is difficult to conclude on that aspect of study.

Table 1: Composition Of Cases

Composition	No. %	Thal. Synd. Cases No. %
Students	475 55.9	38 48.1
Antenatal	361 42.5	27 34.2
Family members	14 1.6	14 17.7
Total	**850 100.0**	**79 100.0**

Thal. Synd.=thalassemia syndrome

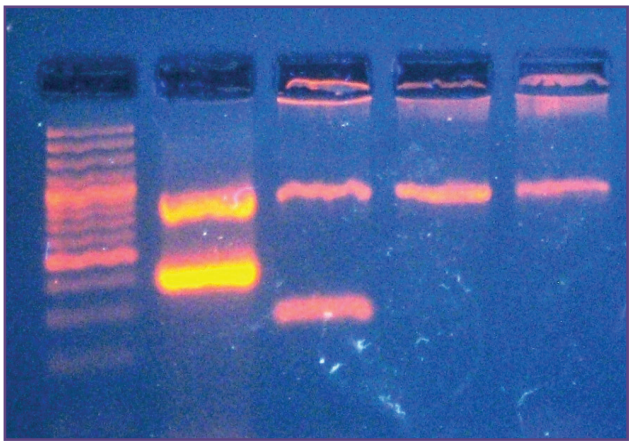
Table 2: Haemoglobinopathy in The Study Groups Subjected to Screening

Haemoglobinopathy	Antenatal n=361 No %	Students n=475 No %	Family n=14 No %	Total n=850 No %
β-TT	22 6.1	36 7.6	11 78.7	69 8.1
TI	--- ---	1 0.2	1 7.1	2 0.2
HbLepore	--- ---	1 0.2	--- ---	1 0.1
HPFH	--- ---	--- ---	1 7.1	1 0.1
HbS	2 0.6	--- ---	--- ---	2 0.2
HbD	1 0.3	--- ---	--- ---	1 0.1
HbE	2 0.6	--- ---	1 7.1	3 0.4
Total	**27 7.5**	**38 8.0**	**14 100**	**79 9.2**

Table 3: Comparison Of RBC Parameters Between Cases of Thalassaemia Syndrome VS Others.

RBC Parameters	Thal. Synd. (MEAN± SD) n = 79	Others (MEAN± SD) n = 771	P Value
AGE	21.7 ± 7.7	21.8 ± 4.1	0.752
Hb (gm%)	10.1 ± 1.5	10.4 ± 1.4	0.085

RBC Parameters	Thal. Synd. (MEAN± SD) n = 79	Others (MEAN± SD) n = 771	P Value
MCV (fl)	68.2 ± 7.1	82.0 ± 10.7	0.000
MCH (pg)	22.1 ± 3.1	28.2 ± 3.6	0.000
MCHC (gm/dl)	31.5 ± 2.0	32.2 ± 1.7	0.562
RDWCV (%)	14.2 ± 1.7	15.8 ± 2.2	0.064
RBC COUNT(million/mm^3)	4.9 ± 0.8	4.0 ± 0.5	0.000
RETIC COUNT (%)	2.9 ± 1.3	1.3 ± 1.1	0.000



Photograph 1: Beta-Thalassemia Mutation Detection by ARMS PCR.

Discussion

The thalassemias, have now become a global problem and common in the Mediterranean region, South- East Asia, the India subcontinent and the Middle East. Approximately 1.5% of the global population are hetrozygotes or carriers of the beta thalassemias and there are significant variations seen even within small geographic regions.[12]

Indian population is ethnically very diverse. The frequency of beta-thalassemia trait has variously been reported from <1% to 17% with an average of 3.3%. Maximum studies that has been done are on small population groups. There is significant variation in the prevalence rate of hemoglobinopathies in different areas and population groups. Punjabi population show a high frequency of HbD, Hb E is more common in eastern region of India and HbS is mainly reported among tribal population.[13]

The multicentric study done in six states of India determining the prevalence of haemoglobinopathies in different groups found overall prevalence of β-thalassemia trait to be 2.78 % and varied from 1.48 to 3.64% in different states, while the prevalence of β-thalassemia trait in 59 ethnic groups varied from 0 to 9.3%. HbE trait was mainly found in Assam (23.9%) and Kolkata in West Bengal (3.92%).[14]

A study was done to evaluate the usefulness of cation exchange high performance liquid chromatography as a tool for detection of haemoglobin variants in a tertiary care centre(AIIMS) in north India and nine additional variants alongwith β- thalassemia were encountered.[15]

Thalassemia Intermedia: Tyagi et al[16] found microcytic hypochromic red cell picture with moderate degree of anisocytosis, poikilocytosis, few fragmented red cells and nucleated red cells, similar to our findings. Tyagi et al[16] found mean HbF to be 46.9 % with values ranging between 18.3- 98.5% and mean HbA_2 of 2.4% ranging between 1.1- 8.4%. Our cases have HbF values 34.1 % and 33.6 % similar to it but slight increased HbA_2 values i.e 11.6 % and 11.4 % respectively.

ThalassemiaTrait : Mean Hb of total cases was 10.1 gm% and ranging from 6.5 gm% to 13.0 gm%. Mean Hb of antenatal group was found to be 9.1 gm % which was slightly lower than the student group 10.9 gm %. In a study done by Gupta et al[17] mean Hb was found to be 9.3 gm%. Lower hemoglobin in some of our cases could be due to associated iron deficiency.

Classical red cell indices for beta thalassemia trait are indicated by MCV < 75 fl and MCH < 27 pg[18]. Mean MCV in our cases was 68.2 fl and mean MCH was 22.1 pg. In one series of 244 cases of beta thalassemia carriers the ,mean MCV was 67 fl and mean MCH was 22.4 pg[123]. George et al[19] found mean MCV to be 64.5 fl and MCH was 21.3 pg with RDW-CV being 15.3 % and mean RBC Count was 5.7 millions/µl. In our cases mean RDW-CV was 14.2 % and mean RBC Count was 4.9 millions/µl. Mean RBC Count in student group was 5.2 millions/µl which is slightly higher than the antenatal group (4.8millions/µl). Their high red cell count corresponded to higher mean Hb of 10.9 gm%.

Microcytosis and hypochromia are commonly observed in the peripheral blood smear in thalassemia trait. Tyagi et al[16] and Gupta et al[17] found microcytic hypochromic blood picture in all the cases of beta-thalassemia trait. We also found microcytic hypochromic blood picture in all thalassemia trait cases. Degree of anisopoikilocytosis also varied from none to mild to moderate degree.

HPLC screening showed constantly elevated HbA_2 in thalassemia carriers in all ethnic groups. HbA_2 in our cases ranged from 3.7 – 6.7 % with a mean of 4.8 %. Mean HbA_2 was 6.1 % as per George et al[19], Tyagi et al[20] diagnosed 31 cases of beta heterozygous thalassemia and HbA_2 levels ranged from 3.9 – 9.0 % with a mean of 6 %. HbF was found to be increased in half of the cases, but values observed in general were in the range of 1-3%[21]. In present study, HbF ranged from 0.1-3.2 % with a mean of 0.8 %.

The identification of beta thalassemia trait is often based on characteristics like higher red cell count, reduced MCV,MCH, raised levels of HbA_2. George et al[19] observed 93.7 of female carriers and 88.9 % of male carriers had a MCV < 70 fl. MCH < 27 pg was found in 25 out of 26 thalassemia carriers in his study. In our study 58% thalassemia trait cases had MCV < 70fl and 42% cases had MCV > 70fl. MCH in our study was found <27 pg in 89.9 % cases and in 7 cases (10.1 %) it was 27.0 pg.

HbE: Homozygotes for HbE are usually asymptomatic and have normal hemoglobin levels but in some cases mild anaemia may be present. The peripheral smear shows microcytosis and increased target cells. Hemoglobin analysis reveals >60 % of HbE levels.[22] In present study hemoglobin level was 10.0 gm% and 11.0 gm% in homozygous HbE and 10.0 gm% in HbE trait. Patients of HbE disease and HbE trait were asymptomatic with hemoglobin levels ranging from 8.8 – 11.8 gm% with a mean of 10.5 gm% in the study of Tyagi et al.[20] In present study, mean MCV was 70.3 fl (59 fl and 76 fl in homozygous and 76 fl in heterozygous) and mean MCH was found to be 23.6 pg (20 pg , 24 pg in homozygous and 27 pg in heterozygous). Absolute values were higher (MCV was 84 fl and MCH was 30 pg) in one study[23] while in concordance with other studies (70.0 fl and 23.6 pg)[24] , Fairbank et al (70.2 fl and 23.6 pg)[25]. In our study red cell morphology was microcytic hypochromic in all 3 cases. Target cells were also found. Ritesh et al found microcytic hypochromic blood picture with target cells in all cases of HbE trait. RBC Count was high in half the HbE cases; RBC Count being 5.7 millions/µl (Fairbank et al) [25] and 5.1 millions/µl (Cunnigham et al)[26]. RBC Count in our cases was 5.1 and 4.9 millions/µl in HbEE and 5.0 millions/µl in HbE trait.

HbE values were 86.5 % and 63.0 % in homozygous and 28.3 % in HbE trait. HbF levels were found to be 1.7 % and 5.2 % in HbE homozygous cases and 2.3 % in HbE heterozygous case. Other workers noted HbE to be 29.4 %[27] , 28.0 % [25] and 27.6 % [24] in HbE trait cases.

Sickle Cell Syndrome : Hashmi et al[28] reported mean age at diagnosis to be 13 years in sickle cell trait cases. As in our cases, Mohanty et al[29] also reported reduced MCV and MCH in sickle cell trait cases(63.5 fl and 19.8 pg). On HPLC analysis we found HbS concentration 34.2 % and 35.0 % respectively, HbA_2 was 3.4 % and 3.5 %, HbF was 1.2 % and 1.0 % respectively.

Our study shows that automated cell counter based parameters and formulae are good, rapid, cheaper and easily available methods for screening of haemoglobinopathies especially for thalassemia trait detection. Screening programmes gives platform for creating awareness and increasing education regarding such disorders in the screened population. We found that hemoglobin disorders has significant percentage in Haryana population so such screening would be of great help in reducing such disorders in homozygous condition by counseling such affected youngsters.

Conclusion

Haemoglobinopathies are most common monogenic disorders of erythrocytes causing high morbidity in afflicted individuals creating financial and psychological burden on relatives of such patients. Automated cell counter based parameters are good, rapid, cheaper and easily available methods for screening of haemoglobinopathies especially for thalassemia trait.

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