Original Article

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Spectrum and hematological profile of hereditary anemia in North Indians: SGPGI experience

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Summary **Thalassemia and other hemoglobinopathies together with red cell enzymopathies are a common cause of anemia, which can be prevented by population screening and genetic counseling. This study was designed to screen the anemic patients for thalassemia, structural hemoglobin variants and red cell enzymopathies. A total of 17047 cases were evaluated from 2009 to 2018 for thalassemia, hemoglobin variants, glucose 6 phosphate dehydrogenase deficiency, pyruvate kinase deficiency and hereditary spherocytosis. Patients' records were entered in a Microsoft excel sheet and a spectrum of disorders was evaluated. Year wise spectrum was also analyzed to see the difference in incidence at different time periods. Incidence of beta thalassemia trait and thalassemia major was found in 11.0% and 3.4% respectively, whereas other hemoglobinopathies were observed in 3.2% of the cases. G6PD deficient cases were 0.2 % and 0.4% had hereditary spherocytosis. No significant difference was observed in incidence of thalassemia and other hemoglobinopathies at different time points. This study provided a health burden and detailed spectrum and prevalence of hemoglobinopathies in North Indians high risk population which contribute toward the development of prevention strategies for better management of hemoglobinopathies. In view of high incidence of thalassemia a routine hematological screening at a primary health center may be introduced as a prospective premarital screening under a thalassemia control program. Moreover rapid and easy quantification of hemoglobin variants (Hb variants) make Cation exchange – High Performance Liquid Chromatography (CE-HPLC) a suitable diagnostic test for the routine investigation of genetic causes of anemia.**

Keywords: Thalassemia, Hb variants, G6PD deficiency, Premarital Screening, HPLC

1. Introduction

The inherited disorders of hemoglobin synthesis are the most common monogenic disorders worldwide. Hemoglobinopathies are a group of inherited disorders of hemoglobin synthesis and a major cause of anemia. Hemoglobinopathies responsible for hemolytic anemia may be divided into two groups. The first one corresponds to thalassemia and the second to the presence of structurally abnormal hemoglobin. Though, there are many variants of thalassemia, β-thalassemia is the most common variant found in the Indian sub-continent. Since thalassemia is difficult to cure, it becomes a priority to prevent this disorder. The clinical spectrum of these disorders varies from asymptomatic conditions (beta-thalassemia minor) to serious disorders such as thalassemia major that require regular blood transfusions and extensive medical care. The only cure for affected children is bone marrow transplantation, which is expensive, risky and difficult to perform with discouraging success rates. The other treatment to sustain life is regular blood transfusion with iron chelation but this is very painful. As anemia is considered a major public health problem in India, so early detection of anemia in these disorders allows timely intervention and prevention from serious consequences.

The most common effective approach for developing countries like India is preventing disorders associated with β-globin gene cluster and major efforts need to be directed at applying a simple and well defined strategy to control these disorders by carrier detection, genetic

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counseling and prenatal diagnosis (*1*). However, accurate and timely detection of various known and unknown Hb variants can prevent occurrence of serious Hb disorders such as thalassemia major in newborns.

Mohanty *et al*. in 2013 (*2*) conducted a multicenter study and after analyzing 56,780 samples from 6 different centers; they reported overall a 2.78% prevalence of thalassemia trait in the studied cohort. They highlight that the prevalence of β-thalassemia varied from 0 to 10.5 % among the different caste/ethnic groups of India (*2*).

Since there is lot of complexity in the Indian population structures and due to high demand for resources; large migration of communities occured in the past from one geographical area to another, and therefore there is a need to evaluate the prevalence of hemoglobin disorders from time to time.

The present study was carried out to determine the prevalence of hemoglobinopathies and β-thalassemia among the North Indian populations from 2009-2018. In addition, our study also aimed to identify rare/unknown Hemoglobin variants by molecular analysis to provide accurate diagnosis and genetic counseling.

2. Materials and Methods

This cross-sectional descriptive study was conducted in Department of Medical Genetics, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow from 2009 to 2018. Individuals with suspected risk of thalassemia and other hemoglobinopathies, with known family history, premarital, preconception (postmarital screening before planning child birth) or antenatal subjects who apparently did not have any suspicion of risk of carrier state and individuals coming voluntarily for screening were included.

To evaluate the cause of anemia a battery of tests related to hematological and biochemical parameters such as Glucose-6-phosphate dehydrogenase deficiency (G6PD), Pyruvate kinase Enzyme Deficiency, Hereditary Spherocytosis and Iron deficiency Anemia (IDA) were carried out.

Four ml blood sample was collected from each individual in ethylene diamine tetra acetic acid (EDTA) and plain vials. Red Cell indices analysis was carried out on a Sysmex 21 automated blood cell counter for red cell indices.

Serum Iron and total iron binding capacities (TIBC) was estimated by the Ferrozine method (International Council for Standardization in Hematology 1990) and using IRON & TIBC kit (Crest Biosystems, India) on the same day of blood collection.

The samples were stored at 4°C and further analyzed in batches for CE-HPLC (Cation exchange – High Performance Liquid Chromatography). CE-HPLC was performed with each blood sample on a BIO-RAD Variant II using beta thalassemia short program pack.

DCIP test was performed for G6PD deficiency. Dichloro phenol indophenols decolorizing test (DCIP Bernstein test) is a screening dye test for G6PD deficiency diagnosis. Nicotinamide adenine dinucleotide phosphate (NADP) reduces the dye DCIP into a colorless state (DCIPH) through the action of G6PD. The rate and the degree of this decolorization are proportional to the G6PD activity in RBCs. Phenazonemethosulphate (PMS) is used as an electron carrier between NADPH and 2-6 DCIP in the test. 20 µL of whole blood followed by 400 µL of DCIP dye solution was mixed in a test tube containg 50 µL of PMS, 25 µL of G6P and 25 µL of NADP and 1 mL of distilled water and overlaid with liquid paraffin incubate at 37°C for 10 min. The mixture color changes from blue to red in presence of G6PD deficiency within 10 min.

Pyruvate kinase (PK) deficiency is due to defects in pyruvate kinase enzyme expression or activity. Pyruvate kinase enzyme catalyzes the last step of glycolysis, the transfer of a phosphate group from phospho(enol) pyruvate (PEP) to ADP, yielding one molecule of pyruvate and one molecule of adenosine triphosphate (ATP). In the pyruvate kinase deficiency test, PEP and ADP were catalyzed by PK to generate pyruvate and ATP. The generated pyruvate is oxidized to produce fluorescence (at 340 nm). The 1000 µL reaction mixture in a tube contained 25 mM potassium phosphate buffer pH 7.4 50 µL, 8 mM magnesium sulfate 100 µL, 0.15mM NADH 100 µL, 0.15 mM phosphoenolpyruvate 30 µL, 0.3 mM ADP 100 µL, and water 620 µL. RBC solution was prepared using packed red blood cells suspended in 20% normal saline. 200 µL reaction mixture was mixed with 20 µL RBC solution in a tube and the mixed solution was spotted on Whatman no 1 filter paper at zero time. Tubes were placed in water bath at 37°C for 10 minutes and again a drop from the mixture was spotted on filter paper after 20 and 30 minutes. In a normal sample disappearance was seen with in 30 min but in pyruvate deficient samples fluorescence takes a longer time to disappear. Since the increase in color or fluorescence intensity is proportional to the increase in pyruvate amount, the PK activity can be accurately measured.

Osmotic fragility is a test to measure the resistance to hemolysis of RBCs exposed to a series of hypotonic solutions. This test is performed to detect thalassemias, red cell membrane disorders and hereditary spherocytosis. In the osmotic fragility test, whole blood is added to varying concentrations of buffered sodium chloride solution and allowed to incubate at room temperature. The amount of hemolysis in each saline concentration is then determined by reading the supernatants on a spectrophotometer at 540 nm. The results of the test may then be graphed, with the percent hemolysis plotted on the vertical axis and the sodium chloride concentration on horizontal axis.

The flow diagram for sample processing is depicted

in Figure 1.

Statistical analysis: Data is presented as number and percentages for discrete variables and as mean ± standard deviation for the continuous variables. The chi square test for trend analysis was done to see the pattern of change of prevalence of different studied causes of hemolytic anemia.

3. Results

Out of 17,047 cases, 3,379 cases were found with normal red cell indices (Table 1). High value of mean corpuscular volume (MCV > 100fl) and mean corpuscular hemoglobin (MCH > 36pg) were found in 211 cases. These 211 cases were found to be Vitamin B12 deficient and excluded for further study. Low Mean corpuscular volume (< 76fl) and Low mean corpuscular hemoglobin (< 26pg) was found in 13,457 cases. Out of these 13,457 samples, 10,201 cases were identified as iron deficiency anemia on the basis of their Serum Iron and Total Iron Binding Capacity (TIBC) levels. G6PD deficiency was found in 37 cases where as hereditary spherocytosis in 75 cases was noted (Table 1). We did not find any case of pyruvate kinase deficiency during the study period.

The remaining samples of 2,607 individuals were further analyzed for thalassemia and other hemoglobinopathies. After doing HPLC 1,764 cases were identified with abnormal hemoglobin variants.

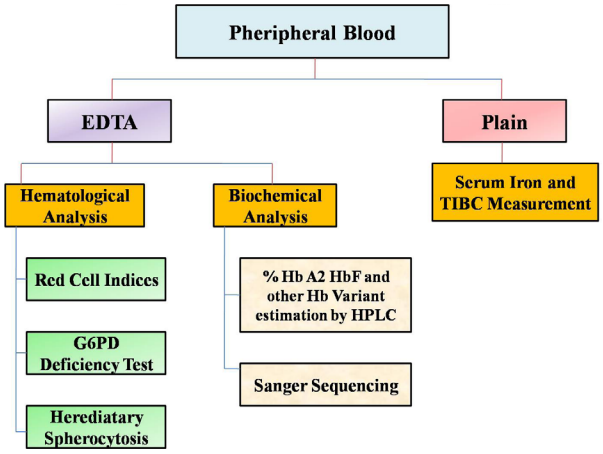

Figure 1. Flow chart showing detail methodology.

These 1,764 cases were further sub characterized on the basis of their clinical and biochemical evaluation like thalassemia major (579) and thalassemia trait (1,185).

Hemoglobin variant disorders found in the present study were HbE trait (204), HbE disease (68), E βthalassemia (51), HbS trait (187), HbS disease (136), Sβ thalassemia (85), HbD trait (85), HbS/HbE Double heterozygous state (17) and HbS/HbD double heterozygous state (34).

Red cell indices were carried out in all samples as this is a primary screening step to find out the cause of anemia. Since thalassemia is a disorder of anemia, hemoglobin values were found reduced in thalassemia along with the microcytic and hypochromic RBCs. Low MCV and low MCH values were recorded in cases as compared to normal. The mean hemoglobin levels, MCV and MCH values of all thalassemia groups are shown in Table 2. MCV and MCH values were lowest in EE group.

HPLC analysis was carried out in subjects with low MCV and MCH and without iron deficiency to find out the status of thalassemia and other hemoglobinopathies. Comparison of mean percentages of various hemoglobins in each thalassemia group are shown in Table 3.

Year wise comparison was done to see whether the prevalence of thalassemia was increasing or decreasing by time and we observed that there was no trend in prevalence of thalassemia and other hemoglobinopathies across different time intervals ($p = 0.826$) (Table 4).

In this study, molecular characterization of beta globin gene sequencing identified a rare HB Köln (HBB:c.295 G>A) and a novel single nucleotide deletion at codon 79 (HBB:c240_240delC) (*3*).

Defining genetic changes will be helpful to explain the clinical findings of the patient and for providing accurate diagnosis and genetic counseling. Therefore, it is important to report rare nucleotide changes in hemoglobin diseases and molecular analysis by Sanger sequencing so that prevalence of various hemoglobin variant can be monitored carefully.

4. Discussion

The present study is an expansion of our previous study published in Prenatal diagnosis (*1*) in which we have done screening of thalassemia and other hemoglobin disorders in extended family members of thalassemia

Table 1. Incidence of different disorders causing anemia in studied cohort

Diagnosis	Male	Female	Total
Normal	2,273 (31%)	1,643 (16.9%)	3,916 (22.9%)
Megaloblastic anemia	94 (1.3%)	117 (1.2%)	211 (1.2%)
Iron deficiency anemia	3,555 (48.5%)	6,646 (68.4%)	10,201 (59.8%)
Hereditary Spherocytosis	37 (0.5%)	38 (0.4%)	75 (0.4%)
G6PD deficient	37 (0.5%)	0	37 (0.2%)
Thalassemia and Hemoglobin variants	1,334 (18.2%)	1,273 (13.1%)	2,607 (15.2%)
Total	7,330 (43%)	9,717 (57%)	17,047

() number in parenthesis represents percentages.

patients, couples coming for premarital screening and college going students. In that study we concluded that a cost effective approach for preventing birth of a thalassemia child is to focus on screening of extended family members rather than screening the general population. We have done a year wise comparison and observed that there is no change in frequency of beta thalassemia and other hemoglobinopathies (Figure 2). Even it is almost more or less from our previous studies.

Table 2. Hematological Parameters in different hemoglobinopathies

Category	Hb (g/dL)	MCV (fl)	MCH (pg)	MCHC (g/dL)
Normal	12.2 ± 2.6	84.5 ± 12.7	29.9 ± 4.6	36.8 ± 2.9
β TT	9.2 ± 2.1	69.2 ± 6.7	20.6 ± 2.8	28.6 ± 2.2
β TM	8.7 ± 2.2	68.7 ± 6.0	21.6 ± 2.9	25.8 ± 4.7
AS	9.1 ± 2.4	68.6 ± 7.0	21.2 ± 3.4	30.9 ± 2.6
SS	8.4 ± 1.6	69.2 ± 7.9	22.3 ± 3.2	30.3 ± 2.4
Sβ	8.3 ± 2.1	68.6 ± 6.2	22.5 ± 3.5	31.1 ± 2.3
S/E	7.4 ± 3.2	67.1 ± 6.9	19.2 ± 4.5	28.4 ± 3.9
AE	8.6 ± 2.4	69.6 ± 6.1	21.1 ± 3.0	29.7 ± 2.7
EE	6.3 ± 2.0	64.1 ± 6.6	18.4 ± 2.5	27.7 ± 2.5
Eβ	6.0 ± 1.4	66.9 ± 4.1	18.9 ± 2.6	26.8 ± 3.3
SD	7.9 ± 1.7	91.3 ± 0.2	32.8 ± 0.6	34.2 ± 4.9
AD	10.4 ± 2.7	69.5 ± 9.7	24.0 ± 4.8	31.2 ± 3.4
DD	8.9 ± 3.0	76.1 ± 12.7	23.7 ± 5.6	31.2 ± 1.6

Table 5 summarizes the various studies conducted in last ten years which have used the patient's selection criterion similar to our study. We can clearly see from the table that the frequency of thalassemia and other hemoglobinopathies varies significantly from one geographical region to another. The figures of World Health Organization (WHO) estimate that approximately 5% of the world's populations are carriers for genetic hemoglobin disorders. Highest (18.1%) prevalence of beta thalassemia trait was observed in New Delhi (*11*) whereas it was found lowest in Dibrugarh, Assam where the HBE was more prevalent (*7*). Sickle cell trait, which was found in high frequency (9.2%) in Maharashtra was found only in 0.8% of studied subjects in the present study. In our other previous studies HBS and HBE was observed in low frequency (*1*).

Colah *et al*. reported that about 1.5% of the world population are carriers of β thalassemia (*12*). In central India the prevalence of β thalassemia trait has been estimated to be 9.59% (*13*). In the present study the most common Hb abnormality detected was β thalassemia trait present in 1185 (11.0%) patients. These data reveal that for the most part of India β thalassemia trait is a common Hb disorder. In this study hematological parameters were used to distinguish cases of β thalassemia trait

Table 3. Comparison of percentage of Hemoglobin variants

Category	HbA	HbA2	HbF	HbD	HbS
NORMAL	80.5 ± 6.9	2.4 ± 0.5	0.8 ± 0.3	-	-
β TT	89.5 ± 2.6	5.5 ± 0.7	0.6 ± 0.4	-	-
β TM	24.6 ± 28.8	3.6 ± 1.3	64.1 ± 28.4	-	-
AS	55.9 ± 5.9	3.4 ± 0.8	2.1 ± 2.3	-	34.0 ± 11.7
SS	3.8 ± 1.7	3.4 ± 0.7	19.0 ± 6.0	-	70.1 ± 8.4
Sβ	3.5 ± 1.7	5.5 ± 0.6	16.9 ± 8.2	-	72.8 ± 8.8
S/E	2.5 ± 0.2	49.8 ±12.6*	4.4 ± 0.5	-	43.7 ± 13.7
AE	48.5 ± 7.1	33.5 ± 11.3*	2.6 ± 7.1	-	-
EE	5.7 ± 2.0	65.4 ± 6.6*	22.8 ± 5.9	-	-
Eβ	4.4 ± 1.4	64.3 ± 14.4*	24.1 ± 8.8	-	-
SD	2.6 ± 0.1	2.3 ± 0.7	16.1 ± 5.7	33.2 ± 9.3	26.3 ± 12.7
AD	56.6 ± 7.8	2.3 ± 0.6	2.5 ± 5.3	33.4 ± 5.2	-
DD	5.8 ± 2.3	3.0 ± 1.2	2.4 ± 2.5	79.8 ± 13.1	-

*HBA2+HBE.

Table 4. Year wise distribution of incidence of anemia causing disorder

Year [May-Apr]	No of Samples screened	Normal	Thalassemia and other hemoglobinopathies	IDA	Other*
2009-2010	1,010	203 (20%)	197 (19.5%)	590 (58.5%)	20 (2%)
2010-2011	1,274	300 (23.5%)	210 (16.4%)	729 (57.2%)	35 (2.7%)
2011-2012	1,471	470 (31.9%)	237 (16.1%)	728 (49.4%)	36 (2.4%)
2012-2013	1,527	358 (23.4%)	229 (14.9%)	899 (58.8%)	41 (2.6%)
2013-2014	2,058	454 (22.0%)	282 (13.7%)	1,279 (62.1%)	43 (2.1%)
2014-2015	2,128	364 (17.1%)	291 (13.6%)	1,444 (67.8%)	29 (1.4%)
2015-2016	2,356	415 (17.6%)	351 (14.8%)	1,554 (65.9%)	36 (1.5%)
2016-2017	2,542	368 (14.5%)	373 (14.6%)	1,745 (68.6%)	56 (2.2%)
2017-2018	2,681	443 (16.5%)	515 (19.2%)	1,674 (62.4%)	49 (1.8%)
TOTAL, 2009-2018	17,047	3,375 (19.8%)	2,685 (15.8%)	10,642 (62.4%)	345 (2.0%)
Chi Square for trend *p* value		< 0.0001	0.0826	< 0.0001	0.038

() number in parenthesis represents percentage.

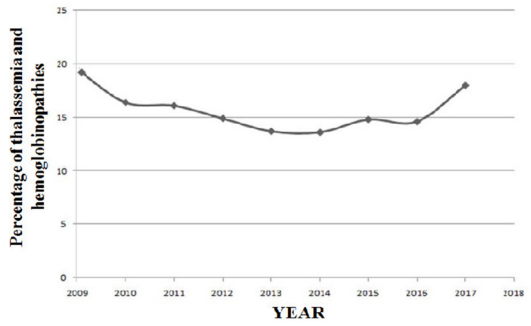

Figure 2. Year wise prevalance of thalassemia and hemoglobinopathies.

from iron deficiency anemia but these parameters were not useful in patients who have both iron deficiency and thalassemia trait. The patients with iron deficiency anemia showed significantly lower levels of HbA2 and MCV than the patients with a normal iron profile. In this study the rate of occurrence of iron deficiency anemia cases increases by year. There is a significant correlation with serum iron level and HbA2 concentration (*14*) and false diagnosis may occur in patients with iron deficiency anemia and borderline HbA2 (3.5-3.9%). Other causes of borderline HbA2 include β thalassemia trait with silent mutation and α triplication (*15*). So it has been emphasized that interpretation of chromatograms must be done after taking into consideration the clinical history, family history, finding of peripheral blood smear and mutation analysis for accurate diagnosis.

Several countries (Italy, Greece, Cyprus, UK, France, Iran, Thailand, Australia, Singapore, Taiwan, Hong Kong, and Cuba) prevent the birth of a thalassemia child up to 70% by setting up comprehensive national prevention programs, which include public awareness and education, carrier screening, and counseling, as well as information on prenatal diagnosis and preimplantation diagnosis (*16-19*). In our country also prevention programs are currently being done but they are limited to certain urban regions and these programs are done by a handful of tertiary care centers. The biggest problem in prevention of thalassemia in India is our traditions for which our society is restricted to an endogamy pattern of marriage and there is some type of social stigma that prevents them from doing thalassemia screening.

This study provided a detailed prevalence and spectrum of hereditary anemia among the North Indian population and will contribute toward the development of prevention strategies and reduction of excessive health care costs in this area, allowing better management of hemoglobinopathies. Also, there is need of a national level prevention program, which registers the thalassemia child, and sets up a national level campaign for public awareness of thalassemia and other hemoglobinopathies.

5. Conclusion

The present study provides a broad overview of the burden

Table 5. Percent prevalence of hemoglobin variants in Indian subcontinent in past 10 years

SR. NO	Year	Name of City/State (*Ref.*)	Total subject	Normal	Anemic	BTT	BTM	AE	EE	E/β THAL	S/E	E/D	AS	SS	S/β THAL	S/D	AD	DD	Others
1	2015	West Bengal (*4*)	10,407	8,898 (85.5%)	1,509 (14.5%)	579 (5.6%)	11 (0.1%)	522 (5.0%)	32 (0.3%)	92 (0.9%)	-	-	37 (0.4%)	7 (0.1%))	4 (0.0%)	1 (0.0%)	7 (0.1%)]	3 [0.0%)	214 (2.0%)
2	2014	Kolkata West Bengal (*5*)	90,210	79,897 (88.6%)	10,313 (11.4%]	3,870 (4.3%)	1,135 (1.2%)	2,418 (2.7%)	358 (0.4%)	1,406 (1.6%)	4 (0.0%)	-	343 (0.3%)	415 (0.5%)	187 (0.2%)	1 (0.0%)	141 (0.2%)	-	35 (0.0%)
3	2014	Burdwan West Bengal (*6*)	1,872	982 (52.5%)	890 (47.5%)	158 (17.8%)	13 (0.7%)	306 (16.3%)	225 (12.0%)	134 (7.2%)	-	-	10 (0.5%)	2 (0.1%)	30 (1.6%)	1 (0.0%)	7 (0.4%)	0 (0.0%)	4 (0.2%)
4	2014	Dibrugarh Assam (*7*)	9,000	3,680 (40.9%)	5,320 (59.1%)	313 (3.4%)	32 (0.3%)	2,294 (25.0%)	1,892 (21.0%)	114 (1.2%)	-	2 (0.0%)	189 (2.1%)	203 (2.2%)	53 (0.0%)	2 (0.0%)	6 (0.0%)	-	220 (4.0%)
5	2013	Gujarat (*8*)	7,000	5,385 (77.0%)	1,615 (23.0%)	839 (12.0%)	292 (4.2%)	11 (0.2%)	10 (0.1%)	4 (0.0%)	-	3 (0.0%)	214 (3.0%)	85 (1.2%)	52 (0.8%)	8 (0.1%)	47 (0.7%)	11 (0.2%)	39 (0.5%)
6	2012	Burdwan West Bengal (*9*)	3,823	2,702 (70.7%)	1,121 (29.3%)	626 (16.4%)	1.6 (2.8%)	176 (4.0%)	7 (0.2%)	134 (3.5%)	3 (0.0%)	-	24 (0.6%)	6 (0.2%)	12 (0.3%)	-	4 (0.1%)	1 (0.0%)	22 (0.6%)
7	2012	Dhule Maharashtra (*10*)	24,723	1,668 (6.7%)	2,726 (11.1%)	183 (0.7%)	3 (0.0%)	3 (0.0%)	-	1 (0.0%)	-	-	2253 (9.2%)	270 (1.1%)	10 (0.0%)	-	3 (0.0%)	-	20,329 (82.2%)
8	2010	New Delhi (*11*)	800	553 (69.1%)	247 (30.9%)	145 (18.1%)	23 (2.9%)	9 (1.1%)	1 (0.1%)	10 (1.3%)	-	-	11 (1.4%)	4 (0.5%)	6 (0.8%)	1 (0.1%)	7 (0.9%)	-	30 (3.7%)
9	Present	Lucknow	17,047	4,092 (24%)	12,955* (76%)	1,185 (11.0%)	579 (3.4%)	204 (1.2%)	68 (0.4%)	51 (0.3%)	17 (0.1%)	-	187 (1.1%)	136 (0.8%)	85 (0.5%)	34 (0.2%)	85 (0.5%)	51 (0.3%)	511 (3.0%)

() number in parenthesis represents percentage. *Hospital referral.

and spectrum of hemoglobinopathies in this region. The size of affected and carriers of hemoglobinopathies in the Indian population is very large. As HPLC is a powerful diagnostic tool for early detection and management of hemoglobinopathies, only a few inconclusive and unidentified cases with coexisting nutritional deficiency require further molecular analysis and iron study. Despite doing a national level screening program, extended member screening for thalassemia, antenatal screening and genetic counseling, we have seen that there is no decline in the birth of thalassemia major in India from the past.

As the centers managing hemoglobinopathies in the high prevalence area increase their experience, more and more families with this condition will come forward and cascade screening and counseling of these families will provide additional cases and carriers, and this will also provide a newer area for research. Therefore, funding by government agencies should be liberal and small scale thalassemia screening industries like laboratories should be established at various states and these centers should be linked to tertiary care hospitals so that mutational and prenatal diagnosis could be treated as prevention programs. In conclusion, a national level awareness program should be performed to prevent the birth of a thalassemia child.

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