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Correlation of Fetal Hemoglobin (HbF) with Clinical Findings and red cell indices in Thalassemia: 'Harbinger of HbF Inducer Therapies'

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ABSTRACT
Aim of this study was to correlate the fetal hemoglobin (HbF) values with red cell indices in patients with thalassemia. Present study was done on 9 patients with thalassemia. All the 9 patients belonged to Tharu Chaudhary community and hailed from mid-western region of Nepal. Thirteen subjects with abnormal hemoglobin structure acted as disease-controls. Eight other subjects with normal hemoglobin structure were also taken as disease-controls. All the subjects (n = 30) included in this study had microcytic hypochromic anemia. Venous blood was collected from all the patients. Blood cells were washed and lysed. Later, Hb electrophoresis was done. Hb electrophoresis revealed presence of HbF in blood of 8 of 9 thalassemic patients. HbF values ranged from 2% to 96% in patients. HbF was inversely correlated with MCHC and MCV. HbA_2 was also correlated with Hct and RBC. Findings of this study suggested that rise in HbF in thalassemic patients may improve hypochromia and thus may favor survival of red cells.

INTRODUCTION

Thalassemic patients have reduced MCV. MCV of 72 fl is maximally sensitive and specific for presumptive diagnosis of thalassemia syndromes. Thalassemias tend to produce a uniform red cell diameter without a concomitant increase in RDW[1]. Studies correlating HbF with red cell indices are few. Therefore, the present study was planned to correlate the level of HbF with red cell indices in thalassemic patients.

MATERIALS AND METHODS

It was planned to investigate the etiopathogenesis of 30 subjects with microcytic hypochromic anemia. Accordingly, the subjects were investigated. The subjects had severe or moderate anemia. Subjects mainly belonged to Banke (n = 16), Bardiya (n = 8) and Dang (n = 4) districts of Nepal. In addition, 1 patient each came from Surkhet and Dhangadi districts. The subjects were clinically examined and findings were recorded on a specially designed proforma. About 2.5 ml of venous blood was collected in EDTA

and blood smears were prepared. Later, detailed hemogram was obtained using automatic cell counter (Nihon Kohden Celltac, E Germany). Total Hb was estimated by cyanmethemoglobin method[2]. Blood smears were stained by Leishman method. Reticulocyte index was calculated[3]. Slide-based sickling test was done using Sodium metasulphite.

Preparation of hemolysate: EDTA blood was washed × 3 with normal saline. The packed blood cells were lysed with distilled H_2O and centrifuged. Supernatant was collected as hemolysate and stored at +4°C.

Fetal hemoglobin estimation: HbF was estimated using 2 different methods, e.g. acid elution cytochemical method[2] and agarose gel electrophoresis at alkaline pH 8.6.

(a) Acid elution cytochemical method: Fresh blood smears were air-dried and fixed in 80% ethanol. Smears were placed in elution solution containing hematoxylin and ferric chloride. Smears were counter-stained with eosin. Later, fetal cells were counted[2].

(b) Agarose gel electrophoresis at alkaline pH 8.6: Five µl of hemolysate was charged into well plates using a unit applicator, the sample was applied into the alkaline hemoglobin agarose gel along with suitable controls and immediately placed in electrophoresis chamber. The sample was electrophoresed at 100V for 45 minutes. The agarose gel was transferred toa developer unit where it was fixed and stained. The equipment was supplied by Biotec-Fischer GMBH Maestro101, Germany. Good separation of HbA, HbF, HbS and HbC was obtained by this method.

On the basis ofabove investigations, 9 subjects were found to be suffering from β thalassemia and they were labeled as Patients. One of the subjects among thepatient group showed an additional fast moving band. Ten of 30 subjects gave positive sickling reaction; these subjects also showed HbS band by hemoglobin electrophoresis. These subjects were diagnosed as having sickle hemoglobin. In addition, 3 other subjects had HbD disease. These 13 subjects were collectively included as Disease-controls with abnormal structure. Eight other subjects had normal Hb structure. These subjects were labeled as Disease-controls with normal Hb structure.

Statistical Analysis: Data was analysed using statistical package for social science for windows (release 17.0). Bivariate analysis was done for negative and positive correlation (r) between variables. Correlation coefficient (r) between two groups was calculated using Karl Pearson's product method. P value of <0.05 was considered to be significant. The values were represented in number, % and mean +S.d.

RESULTS

Patients: Age of the patients ranged from 10 months to 26 (median 4.5) years. Male female ratio was 3.5:1. All the patients belonged to Tharu Chaudhary community. Five patients had fever. Four patients had hepatosplenomegaly. One other patient had splenomegaly alone. One of the patients had jaundice along with fever and weakness.

Results of Hb electrophoresis revealed presence of HbF in blood of 8 of 9 patients. HbF values in patients ranged from 2% to 96.4% (HbF values of <2% were considered as insignificant). High levels of HbF (>12%) were detected in 6 patients. On the basis of results of Hb electrophoresis, 4 of 9 patients were named as β^+ thalassemia major. Hepatosplenomegaly was detected in 2 of 4 patients with β thalassemia major, one patient each had β^+ and β^o disease. Three other patients had β^+ thalassemia minor. One patient each had Sickle cell-β^o thalassemia and β^+ thalassemia intermedia (table 1). These 2 patients also had hepatosplenomegaly.

Disease-controls with abnormal Hbstructure: Two subjects with sickle cell disease produced HbF. One of them with heterozygous sickle cell trait had 16.4% of HbF. Another subject had 0.8% of HbF which was considered insignificant.

Disease-controls with normal Hbstructure: One of 8 subjects belonging to this group

produced HbF; this subject was a 9 months old infant and she was still producing HbF (8.3%).

Red cell indices in 3 groups of subjects: Table 2 shows mean values of red cell indices in 3 groups of subjects. As it will appear, the patients had low total Hb concentration when compared with 2 other groups. Similar low levels were observed in RBC count and MCV in patients when compared with other disease-control groups.

Relation of HbF with red cell indices in patients: Results of estimation of HbF in patients revealed highest HbF level (96.4%) in patient number 9. This patient had total hemoglobin of 8.4 gm/dl, suggesting moderate anemia. Conversely, another patient (no.2) with similar high level of HbF (96.1%) had severe anemia (Hb 1.7 gm/dl). Another patient (no.5) had similar high level of HbF (90.8%) with total Hb of 6.2 gm/dl, suggesting severe anemia. Another patient (no.6) had HbF level of 76.6% with total hemoglobin of 5.3 gm/dl, suggesting severe anemia. It appeared that the patients with high levels of HbF (>70%) had moderate or severe anemia (table 1).Four patients with relatively high levels of HbF (nos. 2,5,6 and 9) had mean MCV of < 67 fl, i.e. 44 fl, 53 fl, 48 fl and 66.3 fl respectively, suggesting inverse relationship. Moreover, patient no.1 with moderately elevated HbF (34%) had MCV of 43 fl, again suggesting an inverse relationship. In contrast to above findings, patient no.3 with HbF of 20.6% had MCV of 73.4 fl. In patient no.4, similar MCV value of 73.5 fl was detected with relatively low HbF of 5.1% (table 1).

Table 3 shows that age was related to Hct and RBC in thalassemic patients ($p < 0.10$) and with Hct in disease-control subjects with abnormal Hb($p< 0.10$).

Table 4 shows that HbA_2 was mildly correlated with Hct and RBC in patients with thalassemia and with MCHC and RDW in disease-control subjects with abnormal Hb.

Table 5 shows results of statistical analysis between red cell indices and HbF in patients. HbF% was found to be significantly correlated with MCHC and also mildly correlated with MCV ($p < 0.10$). Total Hb (gm/dl) also showed some relationship but it could not come upto the level of mild significance ($p < 0.10$). Hence, HbF appeared to have a role in improving RBC indices.

Table 1 : Shows hematological and Hb electrophoresis findings in thalassemic patients.

Patient no. and Mean ±S.d.	Age in years	Total Hbgm /dl	Hct %	RBC million/ mm^3	MCV fl	MCH pg	MCHC gm/dl	RDW %	Reticu-locyte index	Fetal RBC (F cells)	Hemoglobin electrophoresis					Name
											HbA_2 %	Hb S %	HbF %	HbA %	Additional band %	
1	4.5	1.8	7	0.96	42.9	18.8	25.7	27.7	0.5	94	1.5	-	34	64.5	-	β^+Thalassemia major
2	13	1.7	8	0.98	44	19.8	26.5	27.2	0.7	94	1.1	2.8	96.1	-	-	Sickle cell-β^oThalassemia
3	4.5	3.9	12.7	1.73	73.4	22.5	30.7	25.8	0.8	42	1.8	-	20.6	64.5	13.1	β^+Thalassemia intermedia
4	16	11	34.7	4.72	73.5	23.5	32	13.8	0.8	0	0.8	-	5.1	94.1	-	β^+Thalassemia trait minor
5	2.5	6.2	29	3.21	53	21	28	16.1	1.3	98	9.2	-	90.8	-	-	β^oThalassemia major
6	4	5.3	13	2.82	48	22	29	16.8	0.4	96	4.8	-	76.6	18.6	-	β^+Thalassemia major
7	10/12	8.6	26.1	4.02	64.6	21.4	33	17.1	3	54	8.4	-	2	90	-	β^+Thalassemia minor
8	26	10.2	43.5	6.6	65.7	19.9	30.8	18.5	3	2	12.2	-	0	87.8	-	β^+Thalassemia minor
9	13	8.4	36.4	6.48	66.3	29	28	16	1.6	60	3.6	-	96.4	-	-	β^oThalassemia major
Mean	9.36	6.35	23.38	3.50	59.04	21.99	29.3	19.66	1.34	60.0	4.82	-	46.84			
S.d.	8.25	3.24	13.55	2.14	12.18	2.84	2.49	5.56	1.01	39.33	4.15	-	42.58			

"-" in the tables signifies - not detected

Table 2 : Shows mean +S.d. of red cell indices in different groups of subjects.

Red cell indices	Thalassemic patients (n = 9)	Disease-controls with abnormal Hb structure (n = 13)	Disease-controls with normal Hb structure (n = 8)
Hbgm/dl	6.35 + 3.25	8.8 + 2.8	8.75 + 4.35
RBC million/mm^3	3.5 + 2.14	4.17 + 1.7	3.86 + 1.27
MCV fl	59.04 + 12.18	71.9 + 9.5	68.15 + 11
MCH pg	21.99 + 2.84	23.65 + 3.7	22.5 + 5.02
MCHC gm/dl	29.3 + 2.49	31.84 + 3.2	29.23 + 3.47
RDW	19.66 + 5.56	16.93 + 3.01	17.23 + 3.47

Significant differences were not obtained, possibly due to small numbers.

Table 3 : Shows relationship of age with red cell findings and hemoglobin electrophoresis values of product moment correlation coefficient 'r'

Groups	Hb gm/dl	Hct %	RBC Million mm^3	MCV fl	MCH pg	MCHC gm/dl	RDW	Reticulo-cyte index	HbA_2 %	HbF %
Thalassemia patients (n = 9)	0.4698	0.5888*	0.5901*	0.2982	0.0884	0.1056	-0.1534	0.3057	0.1757	-0.2071
Disease-control with abnormal Hb (n = 13)	0.4507	0.5033*	0.2562	0.4514	0.2422	0.2017	-0.3895		0.3735	

* mildly significant ($p<0.10$), other correlations were not found to be statistically significant, most probably due to small sample size (n = 9).
Negative sign (-) shows inverse relationship.

Table 4 shows relationship of hemoglobin A_2% with red cell findings of product moment correlation coefficient 'r'.

Groups	Hb gm/dl	Hct %	RBC million mm^3	MCV fl	MCH pg	MCHC gm%	RDW %
Thalassemia Patients (n = 9)	0.4747	0.5897*	0.5349*	0.1037	-0.1931	0.3327	-0.476
Disease- control with abnormal Hb (n = 13)	0.2522*	-0.3204	0.2096	-0.0129	-0.0018	0.5331*	-0.5793*

* mildly significant ($p< 0.10$). Negative sign (-) shows inverse relationship.

Table 5 : Correlation between HbFVs different red cell indices in patients with thalassemia.

HbFVs Red cell indices	Correlation coefficient 'r'	p value
HbFVsHb	-0.4404	>0.10
HbFVsHct	-0.2645	>0.10
HbFVs RBC	-0.2053	>0.10
HbFVs MCV	-0.5564*	<0.10
HbFVs MCH	0.2784	>0.10
HbFVs MCHC	-0.7177**	<0.05
HbFVs RDW	0.0066	>0.50
HbFVs reticulocyte index	-4449	>0.10

** statistically significant at 5% level of significance ($p <0.05$). Negative sign (-) shows inverse relationship.

DISCUSSION

Present study showed an inverse relationship between HbF levels and total Hb concentrations in 5 of 6 high HbF producing β thalassemic patients. Similar negative correlation was found between HbFVsHct, RBC, MCHC and MCV. Clearly, our findings suggested that patients with moderate and severe anemia had increased levels of HbF. However, in another study involving patients with thalassemia interna, median HbF level was 37.2% and HbF levels correlated positively with total hemoglobin[4].

In 2 patients, mild rise in HbA_2 was seen (9.2% and 12.2%). Mild rise in HbA_2 might have partially improved hypochromia. In our βthalassemic patients, MCH ranged from 18.8 pg to 29 pg, showing direct correlation between HbFVs MCH. In this study, HbF showed positive correlation with RDW in thalassemic patients. In another study, RDW values were higher in patients than in normal controls[1].

These observations supported the contention that HbF might improve the survival of red cells in thalassemia. These findings were consistent with

the results of other studies[5-8]. In addition, growth of *Plasmodium falciparum* is retarded in red cells with fetal hemoglobin resulting in selective advantage for β thalassemia heterozygates in whom postnatal HbF declines at a lower than normal rate[9]. Furthermore, persons with thalassemia are more susceptible to *yersinia enterocolitica* infection[10].

Another important clinical feature of this study was the detection of hepatosplenomegaly in 4 patients. Higher affinity of HbF may lead to increased release of erythropoietin, resulting in development of extra medullary foci of erythropoiesis in liver and spleen. Moreover, accelerated erythropoiesis may also enhance iron absorption[11].

CONCLUSION

Fetal hemoglobin production in β thalassemia may correct hypochromia and may promote survival of red cells. Detection of hepatosplenomegaly in thalassemia syndrome may be due to extra medullary erythropoiesis. Increased erythropoiesis may enhance iron absorption and augment pathology.

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Conflicts of interest: There are no conflicts of interest.

Ethics approval: Present study was approved by the ethical committee of Nepalgunj Medical College, Nepal. Written consent was taken from all the subjects included in this study.

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