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Original Research Article

HBA2 levels in iron deficiency - Can iron deficiency mask thalassemia screening?

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ABSTRACT

Background: Iron deficiency has been found to affect hemoglobin A2 (HbA2) values in HPLC. This can be an issue for thalassemia screening laboratories where there is heavy reliance on increased Hb A2 levels for diagnosis of heterozygous thalassemia state. In resource constrained countries like India this could be real challenging where iron deficiency is widespread and facilities for molecular confirmation in borderline HbA2 values is generally unavailable.

Materials and Methods: It was a prospective study done in a tertiary care center over 18 months. All consecutive patients (n = 164) presenting with microcytic hypochromic anemia on peripheral smear were included for further investigations out of which 92 were found to have pure iron deficiency (Hb < 12 Gm/dL with ferritin less than12 ng/ml) on iron parameters. These patients were divided into two groups, Group A with Hb < 9 g/dl and Group B with Hb > 9 g/dl. Common hematological parameters, iron indices and HbA2 levels were analysed in these two group of patients at baseline and after 3 months of documented oral iron therapy. Chi-square and Pearson tests were used for statistical analysis and a P- value of < 0.05 was considered statistically significant.

Result: As expected iron deficiency was found more prevalent in females (72%) than in males(28%). Mean pre -treatment and post - treatment hemoglobin of patients in group A was 8±0.5 gm/dl and 11.3±1.1gm/dl respectively and in group B was 10.2±0.6 g/dl and 11.5±1 g/dl showing positive correlation. Mean pre treatment and post treatment HbA2 levels of patients in group A were 1.8±0.5% and 2.4±0.5% respectively showing statistically significant change after iron therapy (P< 0.0001) but mean pre treatment and post treatment HbA2 levels of patients in group B were 2.1±0.4% and 2.2±0.5% respectively . this change post therapy was statistically insignificant(P=0.1517).

Conclusion: The change in HbA2 levels was statistically insignificant for patients with mild / moderate iron deficiency anemia (Hb > 9 Gm/DL). Thus diagnosis of β thalassemia trait will not be difficult in patients with concomitant mild iron deficiency anemia but patients with severe iron deficiency anemia should first be treated with iron supplements for correct diagnosis of β Thalassemia trait especially patients with borderline Hb A2 levels.

1. Introduction

The most prevalent causes of microcytic hypochromic anaemia worldwide are iron deficiency and thalassemia.[1,2] It is a clinically important differentiation as one is a deficiency disorder easily corrected by supplements and other is a genetic disorder having long term implications.[3] Although many blood indices were used in past for screening of thalassemia trait, currently cation exchange High performance liquid chromatography (HPLC) is the mainstay of screening and diagnosis of Thalassemia in our country.[4] The simplicity of the automated system

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with internal sample preparation, superior resolution, rapid assay time and accurate quantification/identification of haemoglobin fractions makes this an ideal methodology for routine clinical laboratory.[4,5] Cases which are equivocal or silent on HPLC need molecular genetics (gene sequencing) for confirmation of thalassemia.[6]

High Hb A 2 levels (>3.5%) on HPLC is characteristic of thalassemia and is pivotal for its diagnosis. In heterozygous beta thalassemia (thalassemia trait) this elevation is generally mild in nature (4-9%) while it is greatly increased (> 30%) in homozygous/compound heterzygous beta thalassemia.[7] The various levels of Hb A2 in various clinical conditions are as per Table 1.[8] The technique and equipment used for screening of thalassemia should be sensitive enough to pick up thalassemia trait cases where elevation is only minor. Now, any condition which can lower Hb A2 levels can potentially lead to such cases being missed. Myriad conditions can decrease HbA2 levels eg iron deficiency, anemia of chronic disease, sideroblastic anemia, lead poisoning, juvenile myelomonocytic leukemia, hemoglobin H disease, acute myeloid leukemia, aplastic anemia, and hypothyroidism.[9] However, in community screening scenario such conditions are far and few which generally do not affect screening program significantly. However, Iron deficiency is a rampant health issue in our country and also a potential hindrance to thalassemia trait diagnosis because of lowered Hb A 2 levels. Such missed thalassemia trait cases can be detrimental to national health programs for control of thalassemia.[10]

Iron deficiency has been shown to affect HBA2 levels in previous study which can detrimetaly affect screening of thalassemia trait cases. To best of authors knowledge, no existing Indian studies have studied HbA2 levels iron deficient cases after iron supplementation.[11] The aim of this study is to study HbA2 levels in cases of Pure Iron deficiency Anaemia before and after iron supplements and analyse if iron deficiency significantly mask screening of thalassemia trait cases.

Table 1: Hb HPLC-Hb A2 value interpretation in various clinical conditions[8]

Hb A_2 value	**Interpretation**
0 – 2%	New born
2 - 3.5%	Normal
3.5- 4%	Indeterminate Zone
4.1- 9%	β Thalassemia trait
10 -14%	Hb Lepore(Rare)
22 - 40%	Hb E trait
44-48%	Hb D Iran
70 – 90%	Hb E homozygous

2. Materials and Methods

It was a prospective study conducted over 18 months (Nov 2019 – Jun 2021) at a tertiary care center. All consecutive patients (n = 164) presenting with microcytic hypochromic anemia (Hb <12 Gm/dL, MCV < 75fl) on peripheral smear were included in study for further evaluations. Iron studies (ferritin level, TIBC, and serum iron using Beckman Coulter Access 2 immunoassay) were used to select pure iron deficiency anemia cases. Iron deficiency was defined as ferritin levels less than 12 ng/dL with concurrent high total iron binding capacity (TIBC).[12] Patients with anemia of chronic disorders (n= 29), dimorphic anemia (n=21), microcytic anemia due to other causes (n=22) were excluded from study group. Ninety two (n=92) patients who were found to have pure iron deficiency (Hb < 12 Gm/dL with ferritin less than12 ng/ml) on iron parameters were taken up for further HPLC and followed up till after completion of treatment. These 92 patients were divided into two groups, Group A : Severe Anemia with Hb < 9 g/dl and Group B : Mild/Moderate with Hb > 9 g/dl. Clinical records were accessed through hospital information system. Details of their age, status of Anemia, any coexisting illness, any radiological imaging if done and compliance to treatment was noted. Common hematological parameters and HbA2 levels were analyzed in these two group of patients at baseline and after 3 months of documented oral iron therapy. Hb A2 levels were analyzed using BIORAD D 10 HPLC machine based on cation exchange High performance liquid chromatography. Quality controls were performed before analysis using BIORAD HB A2/F controls. Hb A2 levels in various categories were as follows ; > 4 -9% - diagnostic of Thalassemia trait, 3.5% -3.9% borderline, 2.5%-3.5% - normal, < 2.5% - low HBA2 levels. Borderline cases were repeated after 6 weeks and followed up with gene sequencing studies. All the patients were kept on regular follow up, however 16 patients were lost to follow up. Pre treatment and post treatment parameters were compared and analysed. Chi – square and Pearson tests were used for statistical analysis and a P- value of < 0.05 was considered statistically significant. The study was approved by institute ethics committee and written informed consent of the patients was taken for using their clinical data for study.

3. Results

3.1. Study population

A total of 164 patients presenting with microcytic hypochromic anaemia were segregated based on their hematological/ iron parameters. Patients from both outpatient and inpatient department were included in study. During further evaluation, 72 patients were excluded in cases where cause of microcytic hypochromic anaemia could not be established as iron deficiency. Final study group of 92 patients were followed up post iron

supplementation. The distribution of cases based on age and sex of patients showed that the majority were females in reproductive age group. The mean age of the study populations was 30±17 years with 66 (72%) females and 26 males.

3.2. Clinical features

The study population was divided into two groups based on baseline hemoglobin levels. Group A consisted of patients with severe anemia with hemoglobin <9 gm/dl (n= 25, 27% cases) and Group B with mild/moderate anemia with hemoglobin >9 -12 gm/dl (n=67, 73% cases). Pallor and koilonychia was significantly more common in Group A as compared to group B (p value – 0.0066 & 0.0041 respectively) The patient characteristics and clinical features at baseline are as per Table 2.

3.3. Laboratory investigations

The various baseline laboratory parameters of the two groups were as per Table 3. Significant differences were found in mean Hb levels (P value =0.0001), MCV values (P= 0.0003.), MCH values (p= 0.0071, MCHC values (p=0.0091), ferritin (p= 0.001) and HB A 2 levels (p=0.0031) between the two groups. Post iron supplementation for atleast 2 months the lab parameters were repeated again. A minimum of 60 days of iron therapy was kept as inclusion criteria for the study. We were able to follow up only 76 patients (20 patients in Group A and 56 patients in Group B) and 16 patients were lost to follow up due to ongoing covid pandemic. Mean pre treatment and post treatment HbA2 levels of patients in group A were 1.8±0.5% and 2.4±0.5% respectively showing statistically significant change after iron therapy (P< 0.0001) but mean pre treatment and post treatment HbA2 levels of patients in group B were 2.1±0.4% and 2.2±0.5% respectively which was statistically insignificant (P=0.1517).

4. Discussion

This study was a prospective study done in cases of iron deficient patients with the aim to study effect of iron deficiency on HBA 2 levels in HPLC. Iron deficiency has been found to lower HBA 2 levels and this may mask diagnosis of thalassemia trait in such patients.[11,13] In the past there have been various studies on the same topic with varying conflicting results. Moreover, no previous study has analysed effect of iron replenishment on Hb A2 levels.

We selected 92 cases of pure iron deficiency anemia as confirmed on peripheral smear and iron studies. Our study demographics were comparable with the study done by Sharadamani et al. among rural population showing that most cases of IDA belonged to females in reproductive age group.[14] The author found that majority of patients belonged to age group 21-30 yrs (28.96%) followed by 31-40 yrs (25.09%) with females being 59.8%. This was in concordance with our finding. Pallor could be demonstrated in all the patient except one, in group A and 52 patients (78%) in group B while easy fatigability was present in 20 patients (80%) in group A and 52 patients (78%) in group B. P values between Group A and Group B for pallor was 0.0066 and for easy fatigability was 0.1274 implying that demonstration of pallor is more objective than vague easy fatiguability. It also correlates with severity of anaemia as shown by in a study done by Yavarian et al.[15] Koilonychia and splenomegaly were associated incidental findings with no significant difference was noted in the two groups.

With iron replenishment therapy Hb levels increased significantly in both the groups reconfirming the diagnosis of Iron deficiency Anaemia and role of iron supplements in IDA as shown by other authors Yavarian et al., Wasi et al., Galanello et al. and Rai et al.[15–18] The severity of iron deficiency anaemia can be correlated with levels of MCV, MCH and MCHC, Lower the value- more severe is the IDA. There was a linear correlation of all the three parameters with iron therapy with all values as expected. The same was demonstrated in the studies done by Harthoorn et al., Majid Yavarian et al. and Verma S et al.[15,19,20]

The most commonly used parameters for iron status in body are serum iron levels, TIBC and ferritin levels. We used serum ferritin levels <12 ng/mL as a cutoff for iron deficiency. The iron status in all patients who could be followed up post treatment, as expected, improved after sufficient iron replacement therapy. In our patients, serum iron increased significantly with values reaching the normal range. TIBC, which was high prior to iron therapy, normalized indicating good compliance to iron therapy. Serum ferritin levels improved significantly and reached normal values after iron therapy. These results are similar to previous studies carried out by Sarika Verma et al., Harthoorn et al. and Majid Yavaian et al.[15,19,20]

4.1. HbA2 levels – Pre and post iron supplementation

There have been conflicting reports of the effect of iron deficiency on HbA2 levels. Few authors have reported a significantly lower HbA2 levels in patients with concomitant thalassemia trait and iron deficiency compared to those with isolated thalassemia trait, while others have failed to elicit such a difference. In our study baseline mean Hb A2 levels in both groups were lower than normal (Group A - 1.8±0.5% and Group B (2.1±0.4%) and the difference in values was statistically significantly lower in patients with severe anemia (ie Group A). These patients also showed a significant rise in HBA2 values from 1.8±0.5% to 2.4±0.5% after iron therapy (P < 0.0001). However, in group B mean pre treatment and post treatment HbA2 levels of patients failed to show any significant change (from 2.1±0.4 to 2.2±0.5% with p value=0.1517). In a study conducted by Majid Yavarian et al., the mean HbA2

Table 2: Clinical characteristics of patients in Group A and B

S. No.	Clinical Features	Groups	Present	Absent	P
1	Easy Fatigability	Group A	20(80%)	5(20%)	0.1274
		Group B	52(78%)	15(22%)	
2	Pallor	Group A	24(96%)	1(4%)	0.0066
		Group B	52(78%)	15(22%)	
3	Koilonychias	Group A	3(12%)	22(88%)	0.0041
		Group B	0(0%)	67(100%)	
4	Splenomegaly	Group A	2(8%)	23(92%)	0.110
		Group B	0(0%)	67(100%)	

Table 3: Laboratory characteristics of patients in Group A and B at baseline

S. No.	Clinical Features	Group A	Group B	P
1	Hemoglobin (Gm/DL)	8±0.5	10.2±0.6	0.0001
2	TRBC (million/cmm)	4.1±0.5	4.5±0.4	0.0028
3	MCV(fL)	65±5.2	71.3±4.7	0.0003
4	Mentzer Index (MCV/TRBC)	16±3.2	15.9±2	0.8582
5	MCH (pg)	19.2±2.3	23.4±3.4	0.0071
6	MCHC(gm/dl))	29.6±1.8	31.5±1.5	0.0091
7	RDW (SD-fL)	41.9±3.1	42.4±5.1	0.6474
8	Reticulocyte (%)	1.4±0.9	1.3±0.7	0.5752
9	Iron (μg/dl)	20.4±8.9	23.9±9.7	0.1192
10	TIBC(μg/dl)	629.5±181.6	587.8±147.7	0.2616
11	Ferritin (ng/ml)	6.1±3.5	8.7±3.2	0.001
12	HB A2 levels (%)	1.8±0.5	2.1±0.4	0.0030

Table 4: Comparison of pre and post treatment laboratory parameters in Group A & Group B

S. No	Parameters	Group A			Group B		
		Pretretment (n-25)	Post treatment (n=20)	P	Pre (n=67)	Post (n=56)	P
1.	Hb	8±0.5	11.3±1.1	0.0001	10.2±0.6	11.5±1	0.0001
2.	TRBC	4.1±0.5	4.7±0.3	0.0003	4.5±0.4	4.8±0.2	0.0095
3.	MCV (fl)	65±5.2	78.9±6.1	0.0008	71.3±4.7	77.4±4.6	0.0053
4.	Mentzer Index (MCV/TRBC)	16±3.2	16.7±1.6	0.0840	15.9±2	16.3±1.4	0.1430
5.	MCH (pg)	19.2±2.3	27±3.6	0.0009	23.4±3.4	27.1±3.5	0.0005
6.	MCHC (g/dl)	29.6±1.8	35.5±3.2	0.0001	31.5±1.5	35.6±3.2	0.0084
7.	RDW-SD	41.9±3.1	51.4±5.8	0.0036	42.4±5.1	51.3±5.8	0.0002
8.	Reticulocyte (%)	1.4±0.9	2.2±0.6	0.0092	1.3±0.7	2±0.5	0.0086
9.	Iron(μg/dl)	20.4±8.9	43.6±10.1	0.0001	23.9±9.7	42.8±11.6	0.0094
10.	TIBC(μg/dl)	629.5±181.6	449.9±60.4	0.0087	587.8±147.7	442.3±48.6	0.0029
11.	Ferritin (ng/ml)	6.1±3.5	19.5±3.3	0.0082	8.7±3.2	21.9±5.2	0.0014
12.	HbA2 (%)	1.8±0.5	2.4±0.5	0.0001	2.1±0.4	2.2±0.5	0.1517

levels before and after iron therapy was 2.5% and 3% respectively for men and was 2.1% and 3% for women concluding that iron deficiency does lower HBA2 levels in females with severe anemia.[15] This indicates that while diagnosis of thalassemia trait may be compromised in severe iron deficiency with marked lowering of HBA 2 levels, it is unlikely to affect screening in cases with mild and moderate anemia with only marginal change in HBA2 levels. Our study findings corroborate with Sarika Verma et al. who in their study has highlighted the coexistence of iron deficiency anemia and beta thalassemia trait in Indian patients.[20] The diagnosis of beta thalassemia trait in such patients may be confounded by reduction in HbA2 levels. Hence, iron deficiency should be identified and corrected in patients with high suspicion of beta thalassemia trait, before making any diagnostic or therapeutic decisions based on HbA2 levels. Also, awareness on complications of blood transfusion and hemovisilance should be kept in mind while making the decision.[21]

Post treatment elevation of Hb A2 confirms the effect of iron on Hb A2 levels. Several hypotheses have been put forward for the diminished HbA2 levels in iron deficiency. Wasi et al. have suggested that beta chains may be more competitive than delta chains in heme binding leading to less HbA2 formation in heme deficiency.[16] Lack of iron may also interfere with delta chain synthesis. Other authors have suggested that low HbA2 levels could be due to decreased transcription or translation of delta gene hence interfering with delta chain synthesis and reduced HBA2. Harthoorn- Lasthuizen et al. hypothesized that lack of iron reduces the synthesis of alpha globin chains compared to non alpha chains. With limited supply of beta chains in thalassemia trait, beta chains compete more effectively for alpha chains than delta chains.[19] No previous study has elaborated on this aspect of iron deficiency and Hb A2 levels. This is a critical observation in understanding the pathology behind change in Hb A 2 levels.

5. Limitation of the Study

The major limitation of this study was small study population and short follow up period. For a disease burden of this magnitude, a large population based study with longer follow up is needed for further validation of results. Lastly there are myriad of factors affecting HbA2 levels. A careful history, examination and a detailed laboratory testing may be required to rule out these factors impacting HbA2 levels which are possible in tertiary care centre and can impact the findings of the study.

6. Conclusion

We conclude that iron deficiency anaemia is widespread in our country especially females. Diagnosis of beta thalassemia trait may pose a difficulty in presence of moderate to severe iron deficiency anaemia especially when HbA2 levels are borderline range. Hence, author recommends mandatory screening of individuals suspected of having thalassemia trait for iron deficiency and treating such individuals with iron therapy for atleast 2 months before commenting on HbA2 levels in resource constraint setting like India to avoid missing borderline cases.

7. Source of Funding

None.

8. Conflict of Interest

The authors declare no conflict of interest.

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