Dilemmas in considering β-thalassemia status in subjects with borderline HbA_2 values: a pilot study in Eastern India

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Abstract

Interpreting hemoglobin disorders by high performance liquid chromatography can sometimes deceptive, especially with borderline HbA_2 values. It is often problematic, especially in antenatal cases if the partner is a known thalassemia trait. We tested for underlying β-thalassemia mutations in 24 subjects with borderline HbA_2 values (between 3.0%-4.0%). Amplification refractory mutation system-polymerase chain reaction was used to detect the five common Indian β-thalassemia mutations: [IVS-I-5 (G>C), Cod 15 (G-A), Cod 8/9 (+G), Fr. 41/42 (-TTCT) and Cod 26 (G-A)]. β-*globin* gene sequencing was performed if no mutation was detected. β-*globin* gene defect was not identified in any of the samples. There was no presence of any of the five common mutations in the small cohort. The average value of HbA_2 in 24 normal samples was found to be 3.96. The average values for mean cell volume and mean cell hemoglobin (MCH) were found to be 82 and 28.8 pg respectively. Among these 24 normal samples, 13 had MCH below 27 pg and 11 had MCH above 27 pg. On the contrary, one thalassemic family was screened, in which the father of an HbE-β thalassemia patient was found to have HbA_2 3.1, being a β-thalassemia carrier. Mutation analysis should be offered to all at-risk couples with borderline HbA_2, especially those with values between 3.5% and 4.0% and microcytic hypochromic indices. As, cases with some specific mutational background or clinical condition shows abnormally low HbA_2, so mutation screening should be performed in other partner if one partner found to be carrier or patient of thalassemia.

Introduction

Thalassemia is an autosomal recessive blood disorder. It is caused due to the either decreased or no expression of erythroid globin mRNA of either α or β *globin* gene and subsequent imbalanced in the ratio of α/β globin chain which are clinically manifested by ineffective erythropoiesis and excessive hemolysis.[1] Interpreting and evaluating the abnormal hemoglobins by high performance liquid chromatography (HPLC) is accepted as gold standard as per the guidelines of the World Health Organization for detecting the carrier and disease status for different hemoglobinopathies including thalassemia. In HPLC system (Variant, Bio-Rad Lab., Hercules, CA, USA) hemoglobin (Hb) A_2 co-elutes with HbE at 3.79 (standard deviation +/- 0.01) min retention time within the 6.5 min time scale of β-thal short program. So, in case of HBE carrier or patients, the combined value of HbE and HbA_2 is often in the higher range. But, in case of β-thalassemia carrier or patients HbA_2 values should be greater than 3.5. For people with normal β-globin sequence, the HbA_2 value should be lesser than 4.0. So, the in-between values, which can be considered as borderline HbA_2 values (3.5-4.0 apparently, but strictly 3.2-4.0) generates lots of ambiguity in terms of determining the normal or β-thalassemia carrier or diseased status. In some very rare and exceptional cases, HbA_2 value slightly more than 4.0, can be from a healthy individual, with normal β-*globin* gene sequence. Other instances are also there, though neither very common nor frequent, where a β-thalassemia carrier father, who has an HbE β-thalassemia child, has HbA_2 value 3.1. He would be considered as normal from most of the laboratories, if only HPLC data is observed, though he shows little hypochromic indices [mean cell hemoglobin (MCH)=25.4 pg] with very good hemoglobin (Hb=14.5 g/dL) level (Figure 1) and almost normocytic red blood cells (RBCs) [mean cell volume (MCV)=79.2 fL].

In this paper, we try to emphasize on the notion that, how borderline HbA_2 value (mostly 3.5-4.0) can be deceptive in terms of determining the β-thalassemia diseased or carrier status especially in this mixed population of West Bengal, Eastern part of India with lots of ethnic diversities and migration history.

Materials and Methods

Written consent for evaluation of β-globin mutation was taken from adult participants and in case of children it was taken from the parents as per guidelines of institutional ethical committee. Peripheral blood samples were collected from every participant in the various screening camps, in vials containing 5 mM ethylenediaminetetraacetic acid. The handling of all human blood samples was carried out in accordance with the guidelines established by the Local Ethical Committee.

The initial screening of the participants was done by Hb and complete blood count in automated analyzer (cell counter: Medonic 530; EMerck, Darmstadt, Germany) and finally the β-thalassemia status was confirmed by HPLC. Only those, who were confirmed of their status by these studies, were taken for evaluation for determining the β-thalassemia mutations.

Hematological studies

The participants were evaluated for Hb, MCV, MCH, mean cell hemoglobin concentration, red cell distribution width (RdW), hematocrit. The complete and final screening was done through Hb-variant analysis by HPLC. Hb variants (HbA, HbF and HbA_2/E) were estimated by HPLC (Variant Classic; Bio-Rad Lab.) using manufacturer's protocol.

β-thalassemia mutations

DNA was isolated from white blood cells in the selected cases (N=24), using a DNA isolation kit for mammalian blood (Qiagen, Venlo, The Netherlands). The selected participants were screened for five common β-thalassemia mutations of Eastern India[2,3,4] like IVS1-1 (G-T), IVS1-5 (G-C), codon 8/9 (+G), codon 26 (G-A), and Fr. 41/42 (–TCTT). The screening was performed by polymerase chain reaction

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(PCR) based technique, amplification refractory mutation system (ARMS) as described by Old.[5] Direct DNA sequencing of the β-*globin* gene was also done in all of the selected cases (N=24) for further confirmation of their normal β-*globin* status.

Results and Discussion

As a part of population screening program of Thalassemia Foundation, Kolkata, West Bengal, India, more than 18,000 people were screened for thalassemia and other hemoglobinopathies. From this huge data, we have taken a small population (N=24) with borderline (strictly 3.2-4.0, but mostly 3.5-4.0) HbA_2 value, because we experienced these cases are most ambiguous in terms of determining the β-thalassemia status. This paper comprises of the detailed study of this 24 people, with borderline HbA_2 value, for determining the thalassemia status.

The average value (along with standard deviation) of HbA_2, MCV and MCH in our population (N=2280) with normal β-globin sequence is summarized in Table 1, which is the outcome from population screening program of Thalassemia Foundation, in different districts of West Bengal with almost 15 years duration. This data is given just to show the reference values of standard hematological parameters for individuals with normal β-globin sequence in our population. Within these 2280 normal population the selected group of 24 participants is also included. The average HbA_2 value of these 24 selected participants was found to be 3.96, where as the average value of MCV and MCH was 82 fL and 28.8 pg respectively. Among these 24 normal samples, 13 had MCH below 27 pg and 11 had MCH above 27 pg. The individualistic of HbA_2 value among these 24 participants are given in Figure 2. From there it is quite evident that among these 24 participants, 19 has HbA_2 lesser than 4.0. But, 5 participants show HbA_2 value slightly more than 4.0 (Figure 2). To confirm the β-thalassemia status of these 24 participants, ARMS-PCR was done for five common mutation of this region. No mutation was identified of any of the samples. To confirm the results, β-globin sequencing was also done for all of these participants. But, β-*globin* gene defect or any trace of reportable, so far accepted mutations was not identified in any of the selected 24 samples.

On the contrary, one thalassemic family was screened, in which the father of an HbE-β thalassemia patient was found to have HbA_2 3.1, being a β thalassemia carrier (Figure 1). Mother is HbE carrier and her HbA_2/E value is 29.8. If these couple had went for thalassemia screening, most of the diagnostic facility would have informed them that they would be having normal or HbE carrier child if the diagnostic center only relied on the HPLC data. As, from HPLC data (Figure 1), it is quite evident that the father is normal (HbA_2/E= 3.1%) and the mother is an HbE carrier. But, more comprehensive studies, like screening of common β-globin mutations of the area by ARMS-PCR or determination of mutations, related to thalassemia by direct β-*globin* gene sequencing in the selected ambiguous cases, can help to diagnose the β-thalassemia carrier status with more certainty and clarity, specially in cases

Table 1. Hemoglobin A_2, mean cell volume and mean cell hemoglobin values of males and females with normal β-*globin* gene sequence in Eastern Indian Population (N=2280).

Gender	No. of cases	HbA_2 (%)	MCV (fL)	MCH (pg)
Male	1325	2.8±1.6	74.6±6.7	24.9±3.1
Female	955	2.7±1.4	73.6±15.4	24.9±7.3

HbA_2, hemoglobin A_2; MCV, mean cell volume; MCH, mean cell hemoglobin.

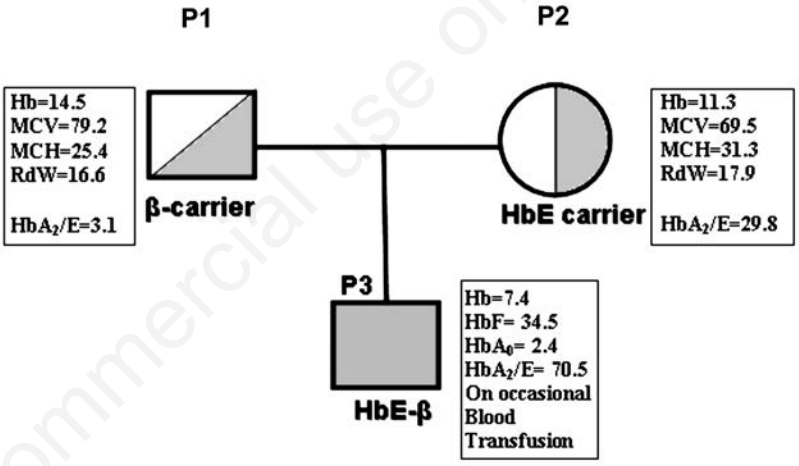

Figure 1. β carrier with normal hemoglobin A_2 (HbA_2) value (3.1%). MCV, mean cell volume; MCH, mean cell hemoglobin; RdW, red cell distribution width.

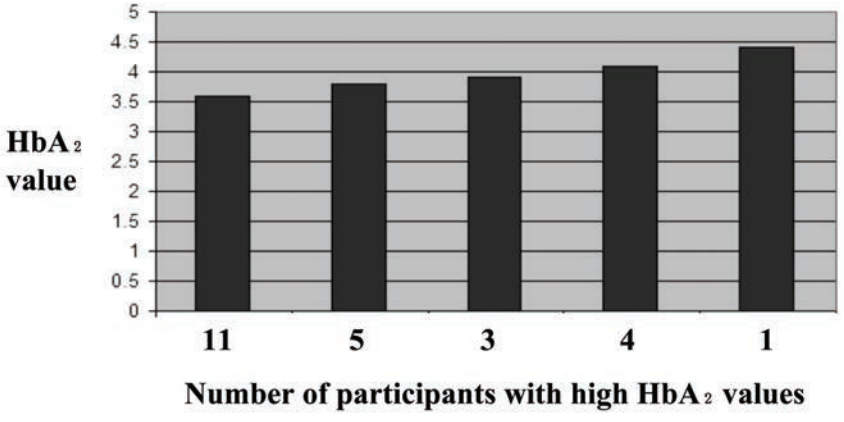

Figure 2. Normal participants with abnormally high hemoglobin A_2 (HbA_2) value (N=24).

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with borderline HbA values, with normocytic to little microcytic and hypochromic RBC indices.

Conclusions

From the above data, we have observed that some cases are not to be found as β-thalassemic even after having the HbA_2/E value equal or slightly more than 4.0. On the contrary, we also have seen a thalassemic family, in which the father of an HbE-β-thalassemia patient was found to have HbA_2 3.1, being a β-thalassemia carrier. These data actually reminds us to consider more comprehensive, careful and adequate strategy for screening of β-thalassemia carrier and/or disease or normal status in population, specially when so many factors like coinheritance of some form of anemia, α-thalassemia, etc. is not rare, different combinations of β-thalassemia specific mutations and β-globin specific allelic variations are frequently present and the population is also very much diverse. But, cost of test should also be a major concern, especially in a developing country like India. So, even if, it is not possible to offer mutational study and direct DNA sequencing of β-*globin* gene to every individual in mass scale screening, but mutation analysis should be offered to all at-risk couples with borderline HbA_2, especially those with values between 3.2% and 4.0% with microcytic, hypochromic indices. As, cases with some specific mutational background or clinical condition show, abnormally low HbA_2, so mutation screening should strictly be performed for the other partner, if one partner already found to be carrier or patient of thalassemia, to avoid the birth of a thalassemia carrier or diseased child.

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