ORIGINAL ARTICLE

Is high pressure liquid chromatography an effective screening tool for characterization of molecular defects in hemoglobinopathies?

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ABSTRACT

Introduction: Hemoglobinopathies constitute entities that are generated by either abnormal hemoglobin or thalassemias. high pressure liquid chromatography (HPLC) is one of the best methods for screening and detection of various hemoglobinopathies but it has intrinsic interpretive problems. The study was designed to evaluate the different mutations seen in cases of hemoglobinopathies and compare the same with screening tests. **Materials and Methods:** 68 patients of hemoglobinopathies were screened by HPLC. Mutation studies in the beta globin gene was performed using the polymerase chain reaction (PCR)-based allele-specific Amplification Refractory Mutation System (ARMS). Molecular analysis for the sickle cell mutation was done by standard methods. **Results:** The IVS 1/5 mutation was the commonest mutation seen and it was seen in 26 (38.23%) of the cases. This was followed by the IVS 1/1, codon 41/42, codon 8/9, del 22 mutation, codon 15 mutation and the -619 bp deletion. No mutation was seen in eight cases. There was a 100% concordance between the sickle cell trait as diagnosed by HPLC and genetic testing. **Discussion and Conclusion:** Our study underlies the importance of molecular testing in all cases of hemoglobinopathies. Although HPLC is a useful screening tool, molecular testing is very useful in accurately diagnosing the mutations. Molecular testing is especially applicable in cases with an abnormal hemoglobin (HbD, HbE and HbS) because there may be a concomitant inheritance of a beta thalassemia mutation. Molecular testing is the gold standard when it comes to the diagnosis of hemoglobinopathies.

KEY WORDS: Hemoglobinopathies, high pressure liquid chromatography, polymerase chain reaction

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INTRODUCTION

Hemoglobinopathies constitute entities that are generated by either abnormal hemoglobin or thalassemias. While abnormal hemoglobin's are caused by a qualitative structural abnormality of the hemoglobin molecule, thalassemias result by diminished synthesis of the globin chain. Patients with hemoglobinopathies have varied clinical presentations of which one of the commonest presentations is microcytic hypochromic anemia.

Hemoglobinopathies are a significant problem in India. The prevalence rate of beta thalassemia mutations in India has been reported to be as high as 17% in some populations.[1] Hemoglobin E-Beta thalassemia is also a very common problem with a high frequency in South East Asia and has been reported from different parts of India.[2,3] Sickle cell anemia is also a common autosomal recessive disorder, which is prevalent in many parts of India.[4,5] HbD has been reported to have a prevalence of about 1.1% (6). The other significant hemoglobinopathies prevalent in India are HbE, sickle cell anemia, and HbD.[2-6]

After presumptive clinical identification of hemoglobinopathies and thalassemia syndromes, screening by HPLC (high pressure liquid chromatography) is done. HPLC is one of the best methods for screening and detection of various hemoglobinopathies and it provides rapid, reproducible, and precise results.[7] However, HPLC is not without intrinsic interpretive problems. A limitation of HPLC is the possibility of alpha thalassemia, normal A2 beta thalassemia, or other hemoglobinopathies that elute with a similar retention values on HPLC which cannot be ruled out.[8] Therefore, for confirmation of the diagnosis and particularly for purposes of genetic counseling, defining the mutation or deletion present may be required. Defining the mutation will also help to evaluate the validity of the screening tests. Depending on the sample size, it will also help to define the prevalence of the mutations in the population.

This was a prospective study which evaluated patients with heterozygous beta thalassemia. In addition, cases that were diagnosed with hemoglobin E, hemoglobin

S, and hemoglobin D disease by HPLC were also included in the study. The cases of heterozygous beta thalassemia were further characterized based on their clinical profile as thalassemia intermedia and thalassemia minor; however, this distinction was not evaluated in the present study. Thalassemia intermedia was defined as a case of thalassemia with clinical severity intermediate between asymptomatic thalassemia minor and transfusion-dependent thalassemia major.[9] By definition, patients of thalassemia intermedia maintain a hemoglobin level of 7-10 g/dl and do not, or only occasionally, require blood transfusion. Thalassemia minor was defined as cases which were clinically asymptomatic but some subjects had moderate anemia.

The study was designed to evaluate the different mutations seen in cases of hemoglobinopathies and compare the same with screening tests. The aim of the study was to validate HPLC as an effective screening tool for screening hemoglobinopathies. We also studied the frequency of the various mutations seen in the Indian population.

MATERIALS AND METHODS

Sixty-eight patients of hemoglobinopathies were evaluated. Initial screening was done by HPLC and the presumptive diagnosis was based on the HPLC findings. HPLC was done using the Broad Variant Hemoglobin testing system. The HPLC results included five cases each of sickle cell disease and HbD disease and three cases of HbE disease. There were 55 cases of heterozygous beta thalassemia. The classification into heterozygous β-thalassemia was based on high performance liquid chromatography and it included the following: HbF <10%, HbA_2 > 3.5%. Demographic data were obtained and a complete blood count (LH-750 analyzer) for red cell indices and a peripheral smear for red blood cell morphology was analysed. Patients with a recent history of transfusion (3 months prior to sample collection) were excluded from the study.

In all cases where there was a suspicion of a hemoglobinopathy, a molecular analysis was performed to detect the mutations. DNA was extracted from the peripheral blood using a standard kit (Qiagen). Mutation studies in the beta globin gene were performed using the PCR (polymerase chain reaction)-based allele specific Amplification Refractory Mutation System (ARMS). This technique uses distinct 3′ specific end primers complementary to either the mutant or the normal gene. Samples were initially screened for the five common mutations. If no mutation was detected in these samples, then they were analysed for the rarer mutations. The details of the mutations and the size of the PCR product is mentioned in Table 1.

Molecular analysis for the sickle cell mutation was done by using primers as described.[10]

STATISTICAL ANALYSIS

Being a descriptive study, statistical analysis was not routinely performed. The descriptive data were expressed in the form of percentages. When statistical analysis was required, the Chi square test was used for comparing the differences between groups. A value of $P < 0.05$ was considered to indicate statistical significance. Statistical analysis was performed using SPSS 13.0 statistical package program (statistical package for social sciences, lLead technologies Inc, USA).

RESULTS

Sixty-eight patients were studied. The age of the patients ranged from a new born baby to 52-year-old male. Thirty females and thirty-eight males were included in the study.

Clinical Phenotype and HPLC Analysis

Of the 68 cases, 5, 3, and 5 patients had HbD, HbE, and HbS disease, respectively. There were 38 cases of thalassemia intermedia and 14 cases of thalassemia minor [Figure 1]. The classification into heterozygous β-thalassemia was based on high performance liquid chromatography and it included the following: HbF <10%, HbA_2 > 3.5%. Three patients remained uncharacterized, one was a new born baby, the second a 3-year-old male and the third was a 27-year-old male.

Spectrum of Mutations in Thalassemia Cases

An analysis of the mutations showed that the IVS 1/5 mutation was the commonest mutation seen and it was seen in 26 (38.23%)

Table 1: Table showing the details of the mutations and the size of the PCR products

Common mutations	
IVS 1 – 5	285 bp
Codon 41/42 (–CTTT)	443 bp
-619 bp deletion	242 bp
IVS 1 – 1 (G – T)	281 bp
Codon 8/9 (+6)	214 bp
Less common mutations	
Codon 16 (–C)	239 bp
Codon 15 (G – A)	500 bp
Cap + 1 (A – C)	586 bp

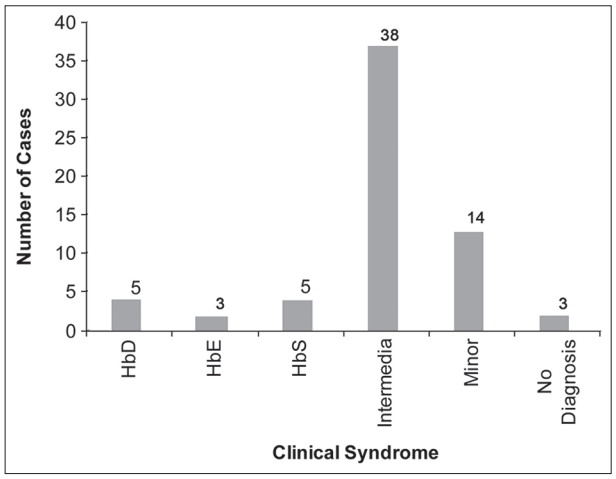

Figure 1: Characterization of the hemoglobinopathies according to the clinical phenotype and HPLC findings

of the cases. This was followed by the IVS 1/1 mutation which was seen in 8 (11.76%) of cases. Codon 8/9 mutation was seen in one case (1.8%) and the codon 41/42 mutation was seen in 8 (11.76%) of the cases. The Del 22 mutation was seen in 7 (10.29%) of patients and the codon 15 mutation was also seen in 7 cases (10.29%). The -619 bp deletion was seen in five cases giving a prevalence of 7.35%. No mutation was seen in eight cases which translate to 11.76% [Figure 2]. If the thalassemia intermedia and thalassemia minor cases are analyzed separately, it is seen that failure to detect a mutation was 5.4% in thalassemia intermedia and 18.75% in thalassemia minor. The difference is statistically significant ($P < 0.05$).

Compound Heterozygotes

Three patients were compound heterozygotes. Two of the patients had co-inherited the IVS 1/1 and IVS 1/5 mutations. Both the patients were females and they were 22 and 23 years old. One patient with heterozygous beta thalassemia had a - 619 del and a del −22 mutation.

Sickle Cell Mutations

Five patients showed a sickle cell mutation. Screening of these patients was suggestive of a sickle cell trait and the PCR RFLP confirmed the diagnosis. Two of these five patients also showed an IVS 1/5 mutation in the beta thalassemia gene.

HbD

Five patients showed the presence of HbD trait on HPLC. Molecular testing was not performed to confirm the diagnosis. However, mutation analysis of these five patients for the presence of beta thalassemia mutations showed mutations in three patients. Of these, one showed an IVS 1/5 mutation and two showed the presence of a Codon 41/42 mutation.

HbE

Three patients showed the presence of an HbE trait on HPLC. No concomitant beta thalassemia mutations were seen in these three patients.

DISCUSSION

In our study, of the 68 cases, 5, 3, and 5 patients had HbD, HbE, and HbS disease, respectively. There were 38 cases of thalassemia intermedia and 14 cases of thalassemia minor [Figure 1]. Three patients remained uncharacterized: One was a new born baby, the second a 3-year-old male, and the third was a 27-year-old male. We report an incidence of mutations in the beta thalassemia gene which closely matches what has been reported earlier. We report that the commonest mutation seen was the IVS 1/5 mutation. Similar results have reported a prevalence varying between 41.34% and 71%.[8,9] The IVS 1/1 mutation was the second most common mutation seen; a similar finding has been reported in studies characterizing mutations in thalassemia intermedia patients.[11] The prevalence of the other mutations correlates well with what has been reported earlier in the Indian literature.[11-14]

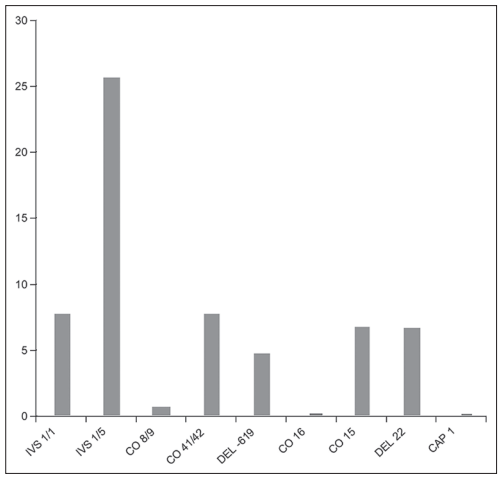

Figure 2: Characterization of the beta thalassemia mutations based on the mutation detected

We had three patients who presented with two mutations (IVS 1/1+ IVS 1/5, −619 del + del −22) and yet had a heterozygous picture on HPLC. It is well known that the severity of beta thalassemia is influenced not only by the types of β thalassemia mutations but also by the expression of α and γ gene expression.[15] Since alpha chain mutations and the Xmn1 polymorphism were not studied, we cannot speculate further on genotype phenotype correlation.

In the present study, no mutation was seen in 11.76% of cases where the HPLC was suggestive of thalassemia intermedia. In a study involving 73 patients with thalassemia intermedia, the characterization of the mutations was possible in 96%, and 4% of the patients remained uncharacterized.[10] After another extensive analysis was carried out and 15 mutations studied, it was found that only 4.5% of mutations remain uncharacterized.[16] The population studied was heterogenous and since we studied only nine mutations in the present study, we believe that a more extensive analysis would characterize most of the mutations.

We also found β thalassemia gene mutations in patients with sickle cell disease and HbD disease. In all these cases, the HPLC did not suggest the presence of a concomitant β thalassemia. Concomitant sickle cell trait and thalassemia is well known and has been reported earlier.[12,17] In our three cases of HbE disease, we did not find any concomitant thalassemia mutation. This is in contrast to the available literature where HbE thalassemia has been reported quite often.[12,17] It is likely that our sample size was not adequate. We did not find much literature relating HbD and thalassemia, and this study has shown that HbD and thalassemia can be co-inherited in up to 60% of cases.

In conclusion, the study underlies the importance of molecular testing in all cases of hemoglobinopathies. Although HPLC is a useful screening tool, molecular testing is very useful in accurately diagnosing the mutations. Molecular testing is especially applicable in cases with an abnormal hemoglobin (HbD, HbE, and HbS) because there may be a concomitant inheritance of a beta thalassemia mutation. As reported,

concomitant mutations act as modifier genes and significantly influence the phenotype.[18] Especially when it comes to prenatal counseling, the phenotype alone should not be a guide to counseling. Molecular testing is the gold standard when it comes to the diagnosis of hemoglobinopathies.

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