ORIGINAL RESEARCH

Rapid Analysis Of Hemoglobin Variants In Beta Thalassemia By HPLC In Northern India.

Nitu Nigam[1], Sanjay Nigam[2], Nidhi Nair[3], Amisha Mishra[4], Yashwant Rao[5], S. K Singh[6]

ABSTRACT

Introduction: Beta-thalassemia is the most common and hereditary blood disorder worldwide, most of which are characterized by base substitution or small deletion or insertion of one or two nucleotides in the globin gene. Today due to the mixture of gene pool, this disorder is now not confined to any particular ethical group/races but each group represents its own sets of mutations. High Performance Liquid Chromatography forms a rapid, sensitive and precise method for detecting abnormal hemoglobin fractions. About 55 cases of Beta-thalassemia have been studied for various hemoglobin variants from Kanpur and adjoining areas.

Material and Method: The study was performed on Agilent 1220 Infinity LC (Agilent Technologies) a High Performance Liquid Chromatography using EZChrom Elite for Beta-thalassemia.

Result: Abnormal hemoglobin variants were analyzed for 55 cases of Beta-thalassemia on High Performance Liquid Chromatography. There were about 18 cases of Beta-thalassemia major and 37 cases of Beta-thalassemia carriers. The frequency observed in our study was HbA_{1c} (0.14), HbF (0.7), HbE (0.45), HbD (0.34), HbS (0.45), and HbA_2 (0.52).

Conclusion: Automated High Performance Liquid Chromatography is an appropriate approach for the screening and presumptive identification of patients as well as carriers of Beta-thalassemia prior to DNA studies for definitive diagnosis.

Keywords: Beta-globin gene, Beta-thalassemia carriers, Beta-thalassemia major, Ethical group, Hemoglobinopathies

How to cite this article: Nitu Nigam, Sanjay Nigam, Nidhi Nair, Amisha Mishra, Yashwant Rao, S. K Singh. Rapid analysis of hemoglobin variants in beta thalassemia by HPLC in northern India. International Journal of Contemporary Medical Research 2015;2(3):544-547

[1]Research Officer, RMCH&RC, [2]Professor, Department of Pathology, RMCH&RC, [3]Research Assistant, RMCH&RC, [4]PhD Student, RMCH&RC, [5]Professor and Head, Department of Pediatrics, GSVM Medical College, [6]Department of Pediatrics, Ursala Horsman Memorial Hospital, Kanpur.

Corresponding author: Dr. Nitu Nigam, PhD (Medical Genetics), Post Doc (Cancer Genetics, USA), In-charge: Central Research Laboratory, Rama Medical College, Hospital and Research Center, Mandhana, Kanpur, UP, India

Conflict of Interest: None

INTRODUCTION

Beta-thalassemia is a heterogeneous group of blood disorder characterized by decreased or absent synthesis of beta globin chain. The disorder may be caused by several molecular defects; most of them are mutations that affect the expression of beta-globin gene. The gene is located on the short arm of chromosome 11p15.5.[1] Phenotypically there are two forms of beta-thalassemia: β°- no β globin chain synthesis and β^+- with some beta globin chain synthesis, clinically presenting as a trait (β° or β^+), thalassemia intermedia (β^+/β^+ or $\beta^+/\beta°$) and beta-thalassemia major (β°/β°).[2] The homozygous state for β° (β°/β°) causes a severe transfusion dependent anemia termed as thalassemia major. Each ethnic group has its own set of mutations that causes beta-thalassemia.[3]

Till date, more than 380 different types of mutations have been recognized that causes this disorder. The endemic area of the disease is the thalassemia belt, which includes the Mediterranean region, part of the Middle-East, the Indian subcontinent & the South East Asia.[2,4] It has been estimated that there are approximately 45 million carriers with the carrier rate of approximately 1: 20 of beta-thalassemia in South Asian countries which include India, Sri Lanka and Pakistan.[5] Treatment of individuals with beta-thalassemia major had to undergo regular blood transfusions and expensive iron chelation therapy which is still not a satisfactory procedure and the disease causes significant morbidity and mortality in affected individuals.[6] The population of Uttar Pradesh is over 200 million and it is a multi-ethnic populated state but marriages most often take place within the same ethnic groups. The facts and figures provided by different reports are useful in establishment of proper molecular screening techniques for the betterment of the thalassemic patients. Such one technique is High Performance Liquid Chromatography that provides specific distribution pattern of hemoglobin variants in patients and carriers that can easily diagnose beta-thalassemia from other forms of hemoglobinopathies.[7]

The present literature indicates needs to evaluate to

control the birth of thalassemia. This will be the first screening center for presumptive identification of patients as well as carriers of beta-thalassemia and to provide molecular diagnosis and prenatal diagnosis in the Kanpur city and the adjoining area. This project is beneficial to the society as it will help in the eradication of the genetic disease like thalassemia.

MATERIAL AND METHODS

Sample Collection Criteria: The sample size consist of 55 cases (18 patients and 37 carriers) with the age ranging between 2 year to 65 year. 2ml of venous blood samples were collected in EDTA vials from each patient and their family members. The patients included in the study were all transfusion dependent. Among them one patient was going through regular iron chelation therapy and another with first time transfusion. Age, sex, history and consanguinity between the parents were recorded. Most of these patients were taking regular transfusion except few cases which had skipped the transfusions at irregular intervals. This was a prospective study carried out in Central Research Laboratory at Rama Medical College-Hospital and Research Center, Rama University, Kanpur for the period of 8 months.

Hematological Parameters: Hemoglobin, Red Blood Cell (RBC) counts & red cell indices were estimated on automated blood counter (Sysmax). Classical red cell indices for beta-thalassemia are indicated by a MCV<75fl (Mean Corpuscular Volume) & MCH (Mean Corpuscular Hemoglobin) <27pg and RBC count >5 million/µl.[8,9]

HPLC Analysis: HbA_2, HbF and other hemoglobin variants were studied by High Performance Liquid Chromatography method used for chromatographic separation of human hemoglobin.[10,11,12] We have used Agilent 1220 LC (Agilent Technologies) a High Performance Liquid Chromatography using EZChrom Elite for beta-thalassemia. The beta-thalassemia kit (Gordion Diagnostik, Turkey) was used for analyzing all the samples. A chromatogram of the cases with beta-thalassemia major detected by the EZChrom program are detected by 1220 LC (Agilent Technologies).

Principle: Agilent 1220 LC is a fully automated High Performance Liquid Chromatography system which uses double wavelength detection of 415nm & 600nm. Several elution methods including specific column, buffers and software were available from manufacturer (Gordion Diagnostiks, Turkey). The EZChrom Elite for beta-thalassemia has been designed to separate and determine the retention time and area percent of HbA_2, HbF & other Hb variants and provide quantitative determination of abnormal hemoglobin. In the 3 x 0.46 cm nonporous Cation-Exchange column, the elution occurs at the flow rate of 1.5ml/min with an analytical time 6.5 minutes by a gradient of buffers A & B that differ in pH and ionic strength. The 5µl of whole blood sample was taken from each case in eppendroff and 1ml of lysis buffer was added to each. After 10 minutes of incubation at room temperature, 10µl of mixture was taken and was injected.

Interpretation of Reports: Reports and chromatographs generated were studied and interpreted by observing the HbF & HbA_2 levels for beta-thalassemia and retention time & area percentage was analyzed for other structural variants. Each chromatograph shows the peak of HbA_{1c}, HbD, HbC, HbE, HbS & HbA_0. Retention time for each hemoglobin variant was interpreted according to the values provided in the manufacturer guide for variants.

RESULT

Around 55 cases were taken into account for study, in which 18 cases were beta-thalassemia major and 37 were carriers. Carriers were family members of the patients. All the cases analyzed were detected with abnormal hemoglobin variants in the study. Presumptive identification of hemoglobin variants was based on retention time and area percentage as shown in Figure-I.

However geographical factor, ethnicity and clinical presentation were also taken into consideration. Frequency of hemoglobin variants identified in analysis is shown in Table-I and Table-II shows the relevant Red Blood Cell (RBC) parameters of beta-thalassemia majors and carriers.

DISCUSSION

In recent years, High Performance Liquid Chromatography (HPLC) has become a quick reference method for the study of hemoglobin (Hb) abnormalities and beta-thalassemia. Cation-exchange HPLC is the method of choice to quantify normal and abnormal Hb fractions.[11-15] India is an ethnically diverse country with marked regional variations. This diversity is reflected in the presence of different regions. Due to migration, there is a constant mixing of peoples from different regions. Many of these abnormal variants are in heterozygous state, but when combined with other variants they may give rise to severe disorder. Therefore, there is always a need for screening method to detect maximum variants and HPLC has the advantage of quantifying HbF, HbA_{1c}, and HbA_0 along with detecting other variants in a single screening test.[16] HPLC is a sensitive, specific and

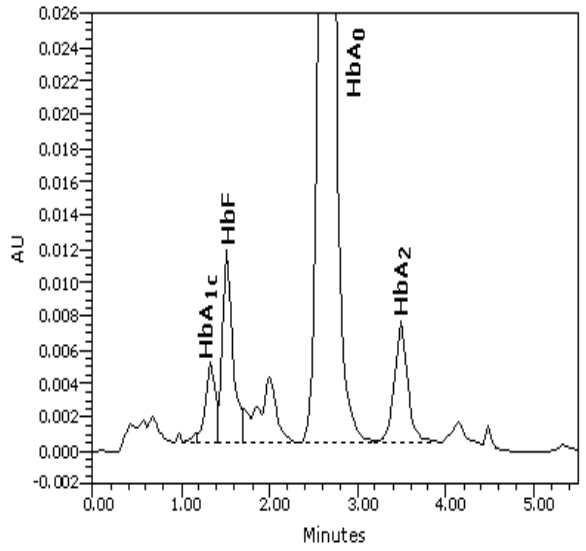

Figure-I: Beta Thalassemia Variant Chromatograph By EZChrom Elite on Agilent 1220 Infinity LC
The above figure shows a typical chromatograph dipicting HbA_{1c}, HbF, HbA_0 and HbA_2 peaks obtained using EZChrom Elite software on Agilent 1220 infinity LC HPLC system.

Hemoglobin variants	Number (%)
HbA_{1c}	8 (0.14)
HbF	39 (0.70)
HbA_0	26 (0.47)
HbE	25 (0.45)
HbA_2	29 (0.52)
HbD	19 (0.34)
HbS	25 (0.45)
HbC	25 (0.45)

Table-I: Frequency of hemoglobin variants identified in analysis

N= 55		
Beta-thalassemia Major (n=18) Mean ± 2S.D		**Beta-thalassemia** Carriers (n=37) Mean ± 2S.D
Hb	4.31 ± 0.23	10.83 ± 0.88 gms/dl
RBC	3.15 ± 0.48	5.1 ± 0.34 X 10^{12}/L
MCV	58.55 ± 2.08	74.32 ± 4.48 fl
MCH	14.94 ± 0.88	20.97 ± 0.28 pg
MCHC	26.61 ± 2.16	k26.83 ± 1.58 gms/dl

Table-II: Hemoglobin (Hb), Red blood cell (RBC) count, Red cell indices (MCV, MCH, MCHC) in β thalassemia majors and carriers.
Here N stands for Total number of patients; MCV for Mean Corpuscular Volume; MCH for Mean Corpuscular Hemoglobin and MCHC for Mean Corpuscular Hemoglobin Concentration.

reproducible and less time consuming method, hence it is an ideal for routine clinical laboratory analysis of thalassemia screening.[17] Beta-thalassemia, being the major concerns of our study so quantization of HbA_2 and HbF levels by HPLC was of prime importance in our laboratory where facilities for genetic studies are available.

Identification of beta-thalassemia is often presumptively based on a characteristic of red blood cell count, red cell indices, and raised levels of HbA_2 and unbalanced globin chain synthesis in an individual of an appropriate ethnic origin. Family studies are of importance in elucidating this inherited disorder of Hb synthesis.[8] Beta-thalassemia trait formed the largest sub group of abnormal hemoglobin (8.9%). The characteristic hematological findings in a typical case of beta-thalassemia trait include microcytosis with raised RBC counts. Hemoglobin rate is quite reduced than normal. The mutations common in an Indian setting include IVS1-5(G-C), 619 bp deletion, IVS 1-1(GT), CD8/9(+G), CD41/42 (-CTTT), CD15 (G-A), CD30 (G-C).[18] HbA2 levels >7% are usually seen with being the major concern in this study. Quantification of HbA_2 and HbF along with other variant levels by HPLC was of prime importance in our laboratory where facilities for genetic studies for thalassemia are available. In our study HbF frequency was higher 39 (0.70) in both beta-thalassemia major and carrier. Presence of HbF and HbA_2 is useful for detection of homozygous beta-thalassemia variants. HbA_0 and HbA_2 traits were found in 26 cases (0.47) and 29 cases (0.52) respectively.

Detection of other variants becomes important due to complex interactions in cases with double heterozygous and homozygous states, which may lead to severe hematological abnormalities. Findings must be supplemented by hemogram findings, family/ sibling studies, hemoglobin electrophoresis, other confirmatory techniques and molecular studies based on HPLC findings and on a case-to-case basis.

CONCLUSION

The hallmark of classical beta-thalassemia is the presence of an elevation of HbA_2 and HbF, where the recommended method of measurement is done by automated HPLC. HPLC forms a rapid, accurate and reproducible tool for early detection and management of hemoglobinopathies and thalassemia. This is especially important in view of high incidence of beta-thalassemia. Early detection of traits will prevent occurrence of thalassemia major in offspring. Detection of other variants becomes important due to complex interaction in cases with heterozygous and homozygous states which may lead to severe hematological abnormalities. Due to high prevalence of Hemoglobin disorders, premarital screening routinely has been done for prevention of high risk

marriages. The present study conducted using HPLC reflects the magnitude of hemoglobinopathies and thalassemia in a hospital based small population which may be in fact the tip of an iceberg, but this type of study can definitely help to increase awareness among patients suffering from these disorders.

REFERENCES

1. Danjou F, Anni F and Galanello R. Beta-thalassemia: from genotype to phenotype. Haematologica. 2001;96:1573-575.
2. Weatherall DJ, Clegg JB. Public health reviews: Inherited hemoglobin disorders: an increasing global health problem. Bull World Health Organ 2001;79:704-12.
3. Brozovic M, Henthorn J, Investigation of abnormal haemoglobins and thalassemia in Dacie JV & Lewis SM, eds. Practical Haematology. Churchill Livingstone, London, p.g. 249.
4. Gupta A, Hattori Y and Agarwal S. Initiation codon mutation in an Asian Indian family. Am J Hematol 2002;71(2):134-36.
5. Agarwal S, Gupta A, Gupta UR, Sarwai S, Phadke S and Agarwal SS. Prenatal diagnosis in beta-thalassemia: an Indian experience. Fetal Diagnosis and Therapy 2003;18:328-32.
6. Nigam N, Munshi N, Patel M and Soni A. Distribution of beta thalassemia mutation and its correlation with alpha thalassemia in Gujarati families. Int J Hum Genet. 2003;3:221–224.
7. Christopher FA, Kumari A and Agarwal CS. Unique pattern of mutations in β-thalassemia patients in Western Uttar Pradesh. Indian J Hum Genet. 2013;19:207-12.
8. Rund D, Filon D, Strauss N, Rachmilewitz EA, and Oppenheim A. Mean corpuscular volume of heterozygotes for beta-thalassemia correlates with the severity of mutations. Blood. 1992;79:238-43.
9. George E, Jamal AR, Khalid F and Osman KA. High Performance Liquid Chromatography (HPLC) as a screening tool for classical beta-thalassemia trait in Malaysia. Malays J Med Sci. 2001;8:40-46.
10. Ou CN, Buffone GJ, Reimer GL and Alpert AJ. High Performance Liquid Chromatography of human hemoglobins on a new cation exchanger. J Chromatogr. 1983; 266:197-205.
11. Kutlar A, Kutlar F and Wilson BJ. Quantitative of hemoglobin components by high performance cation-exchange liquid chromatography: it is use in diagnosis and in the assessment of cellular distribution of hemoglobin variants. Am J Hematol. 1984;17:39-53.
12. Joutovsky A, Hadzi-Nesic J and Nardi AM. HPLC retension time as a diagnostic tool for hemoglobin variants & hemoglobinopathies: a study of 60000 samples in a clinical diagnostic laboratory. Clin Chem. 2004;50:1736-747.
13. Wilson JB, Headlee ME and Huisman THJ. A new high performance liquid chromatographic procedures for separation & quantitation of various hemoglobin variants in aldults and new born babies. J Lab Clin Med. 1983;102:174-85.
14. Rogers BB, Wessels RA, Ou CN and Buffone GJ. High Performance Liquid Chromatography in the diagnosis of hemoglobinopathies and thalassemia. Am J Clin Path. 1985;84:671-74.
15. Samperi P, Mancuso GR, Dibenedetto SP, Di Cataldo A, Ragusa R and Schilirò G. High performance liquid chromatography (HPLC): a simple method to quantify HbC, O-Arab, Agenogi and F. Clin Lab Hematol.1990;13: 169-75.
16. Mohanty D, Colah RB, Gorakshakar AC, Patel RZ, Master DC, Mahanta J, et al. Prevalance of beta-thalassemia & other hemoglobinopathies in six cities in India: a multicenter study. J Community Genet. 2013;4:33-42.
17. Pant L, Kalita D, Singh S, Kudesia M, Mendiratta S, Mittal M, et al. Detection of abnormal hemoglobin variants by HPLC method: Common Problems with suggested solutions. International Scholarly Research Notices 2014;2014(2014):10.
18. Sood SK, Madan N, Colah R, Sharma S, Apte SV and editors. Collaborative study on Thalassemia. Report of ICMR task force study. ICMR. 1993;53