RESEARCH ARTICLE

Molecular Characterization of Haemoglobin E

Sara Ejaz,[1] Ghulam Mustafa,[2] Shagufta Khaliq,[3] Shahida Mohsin[4]

Abstract

Objective: To detect mutation in cases having haemoglobin A2 level >7% on high performance liquid chromatography.

Method: The cross-sectional, descriptive study was conducted from July 2017 to December 2018 at the Department of Haematology and Human Genetics and Molecular Biology, University of Health Sciences, Lahore, Pakistan, and comprised patients of either gender with haemoglobin A2 ≥7%. The samples were collected from different cities of Punjab in collaboration with the Punjab Thalassemia Prevention Programme, Lahore. The samples were subjected to complete blood count and high performance liquid chromatography using automated haematology analysers and variant-II beta thalassemia short programme, respectively. To analyse haemoglobin E mutations at the molecular level, polymerase chain reaction-restriction fragment length polymorphism (PCR_RFLP) was performed using a type IIS restriction endonuclease known as MnlI (derived from Moraxella nonliquefaciens) to cleave DNA at specific sites and the results were further confirmed on randomly selected samples using Sanger sequencing. Data was analysed using SPSS 25.

Results: Of the 39 patients, 15(38.5%) were males and 24(61.5%) were females. The overall median age was 14 (23) years. There were 29 (74.4%) patients with thalassemia family history, and 22(56.4%) had a positive family history of transfusion related to thalassemia, while no patient had a family history of iron therapy. The median haemoglobin A, haemoglobin A2 and haemoglobin F levels were 72.2 (65.2-79.1) %, 26.6 (19.1-34.0) % and 0.9 (-0.8-2.6) %, respectively. After molecular investigation, HbAE mutation was found in 23(59%) patients, while wild type HbAA genotype was found in 16(41%). The heterozygous HbE mutation was present in 23(59%) patients.

Conclusions: Frequently missed/undiagnosed cases of haemoglobin E that co-elute with haemoglobin A2 in the same high performance liquid chromatography window were detected among those with haemoglobin A2 ≥7%.

Key Words: Haemoglobin E, Beta-thalassemia, High performance liquid chromatography, Restriction fragment length polymorphism. (JPMA 73: 37; 2357) **DOI: 10.47391/JPMA.7138**

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Introduction

Haemoglobinopathies are the most common inherited single-gene disorder in which the haemoglobin (Hb) molecule produces abnormal globin chains. Pathophysiology of the disease includes quantitative or qualitative abnormalities. The presence of aberrant globin chain structure characterises the qualitative abnormalities known as Hb variants. Thalassemias are characterised by a decreased amount of normal globin chain formation and are classified as quantitative deficiencies.[1] The world has a high prevalence of haemoglobinopathies. Each year, an estimated 300,000-400,000 children are born with a severe hereditary Hb disease, with roughly 90% of them occurring in low- and middle-income countries (LMICs).[2] Because of Pakistan's location in South Asia, hemoglobinopathies and Hb diseases, like HbD, HbE and HbS, are common there, either on their own or in combination with beta thalassemia.[3] Some of these Hb abnormalities, like homozygous HbEE or heterozygous HbAE or E-trait, are clinically asymptomatic and are linked to either mild to moderate or no anaemia, respectively. However, HbE/beta thalassemia showed a remarkable range in the severity of its clinical manifestations, ranging from a mildly asymptomatic state (e.g. heterozygotes, HbAE) to a mild to moderate state (e.g. homozygotes HbEE) to a severe anaemia that required transfusions (e.g. HbE with β°-thalassaemia). HbE/HbS causes sickle cell disease, which is comparable to sickle/beta+thalassemia.[4]

HbE is a frequent beta-globin chain variant that occurs when glutamine is replaced by lysine at codon 26 of the beta-globin gene. In both homozygous EE and heterozygous EA or E-trait forms, HbE can co-inherit with

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alpha, beta, HbS, HbC, and other Hb variants. HbE trait is clinically silent; HbEE is associated with mild or moderate anaemia.[5] The clinical severity of HbE/beta thalassemia, however, ranged from a moderate to severe condition that required transfusions. HbE/β+ thalassemia is milder in severity than HbE/β° thalassemia which is a transfusion-dependent condition. As beta thalassemia is extensively prevalent in Pakistani population with the carrier rate of 5-7%,[6] the frequency of HbE/beta-thalassemia is expected to be high. HbE/beta-thalassemia patients make up over 50% of those with severe beta thalassemia around the world.[7]

Heterozygous for the HbE phenotype (HbAE) are two adult beta globin genes; one normally found and one mutant. According to clinical data, people with HbE heterozygous or E-trait live normal and healthy lives. The homozygous presence of the HbE gene causes HbE disease. In HbE disease, both of the normal beta globin genes are replaced by the HbE gene. Homozygotes HbEE exhibit a little globin-chain imbalance similar to thalassemia heterozygotes. In the homozygous state, HbEE is a benign condition that does not cause any clinical symptoms, therefore the affected individual can lead a normal life.[8] When the HbE gene is acquired in combination with either the β0 or β+ Hb gene, alpha thalassemia, or any other Hb variants, like HbS, it is known as a compound heterozygous condition. The existence of haemoglobinopathies, which is present along with the HbE gene, determines the clinical appearance of this compound type.[9] In vitro, at least, HbE shows increased sensitivity to oxidants and appears to be fairly unstable. In vitro studies of Hb production do not show instability similar to that observed in other unstable Hb variants despite the fact that HbE is unstable at higher temperatures, such as those that may occur in a number of viral illnesses.[10]

Early diagnosis of HbE is crucial because the carrier rate of HbE in one Pakistani population was estimated to be 1.9%[3]. When paired with beta-thalassemia or HbS, this variant causes mild to severe disease. The first tools used to investigate haemoglobinopathies are complete blood count (CBC), red cell indices, and peripheral film assessment. Based on these findings, high performance liquid chromatography (HPLC) is the most practical and extensively used technology in Pakistan, but it does have certain drawbacks. These include identifying Hb variants with the same electrophoretic mobility as S/D/G/Q/Lepore and A2/E/C, as well as detecting compound heterozygous variants, such as HbS/HbC, HbS/HbD, HbD/HbE, HbE/thal, and HbD/thal. For this reason, molecular techniques, like polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and gene sequencing are carried out in order to arrive at a conclusive diagnosis.[11,12]

The current study was planned to detect mutation in cases having HbA2 level >7% on HPLC. with the aim of drawing attention to the frequently missed/undiagnosed cases of HbE at the molecular level.

Subjects and Methods

The cross-sectional, descriptive study was conducted from July 2017 to December 2018 at the Department of Haematology and Human Genetics and the Department of Molecular Biology, University of Health Sciences, Lahore, Pakistan. The samples were collected from different cities of Punjab in collaboration with the Punjab Thalassemia Prevention Programme (PTPP), Lahore.

Those included using non-probability convenience sampling technique were patients of either gender aged 2 years or more with microcytic hypochromic anaemia (mean corpuscular volume [MCV] <80fl, mean corpuscular Hb (MCH) <27pg, and red blood cell (RBC) count >4.5 million/l) and HbA2 ≥7%. Patients having undergone blood transfusions within the preceding three months, those who were diabetics, and those on iron replacement therapy were excluded.

The sample size was calculated using the World Health

$$n = \frac{Z^2_{1-\alpha/2}\, P\,(1-P)}{d^2}$$

Organisation (WHO) formula for health studies version 2.0.21.[13]

Within the formula, 〖Z^2〗_(1-α/2) = for 95% confidence level was 1.96; P was anticipated proportion which was taken as 0.36%[14]; And d was margin of error 2%.

The approval was obtained from the ethical review committee and the advanced studies and research board (ASRB) of the UHS. Informed consent was taken from all the patients before taking 3ml of blood samples in ethylenediaminetetraacetic acid (EDTA) vacutainers. The samples were subjected to testing at the institutional laboratory as well as the PTPP laboratory at the Sir Ganga Ram Hospital, Lahore. A fully automated system (Sysmex XT-1800i, Japan) was used for CBC. An HPLC automated system (Variant-II β-thalassemia Short Programme Bio-

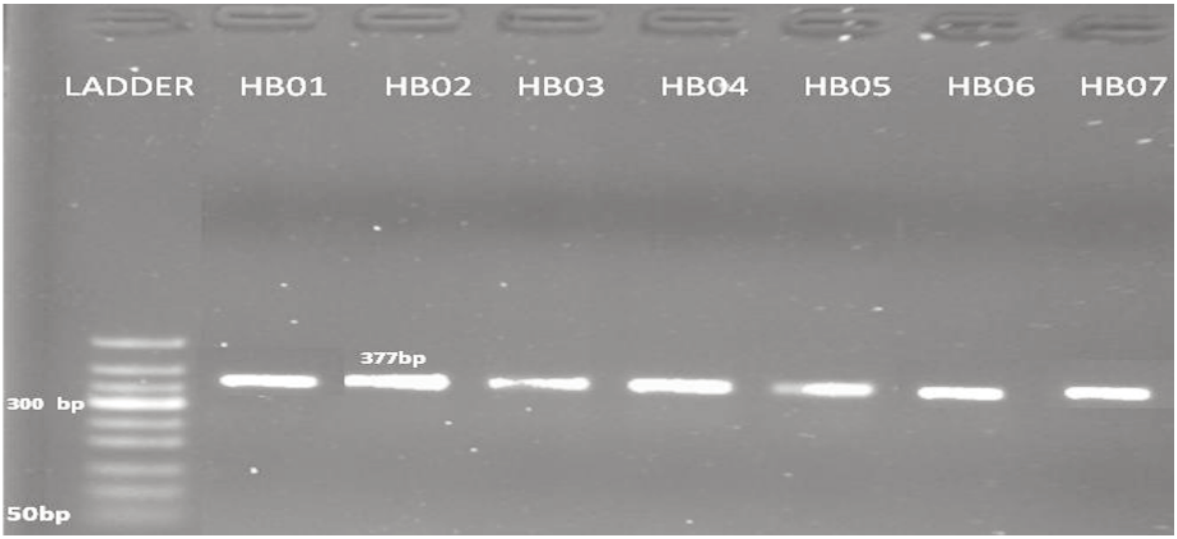

Figure-1: Polymerase chain reaction (PCR) gel picture showing the 377-bp amplification product. The image was taken after loading the PCR product onto a 2.5% agarose gel and staining it with ethidium bromide.

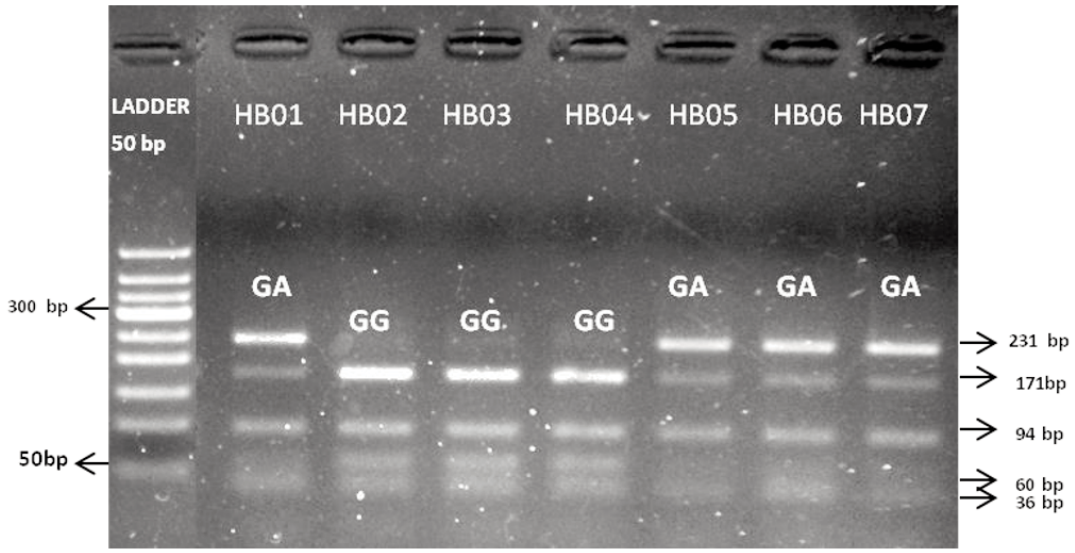

Figure-2: Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) gel image showing 5 deoxyribonucleic acid (DNA) fragments (GA genotype) in samples # HB01, HB05, HB06 and HB07, and 4 DNA fragments (GG genotype) in samples # HB02, HB03 and HB04, indicating haemoglobin (Hb) AE and AA phenotypes, respectively.

Rad), was used to isolate different Hb variants. For HbE molecular characterisation, PCR-RFLP technique was used.

Deoxyribonucleic acid (DNA) of all patients was extracted from venous blood samples using the phenol-chloroform extraction procedure, and PCR was performed to amplify the targetted region of the beta-globin gene, which included exon I, the first intervening sequence (IVS-1), and part of exon 2, for HbE genotyping. The primer was optimised and PCR reactions were carried out under ideal circumstances to obtain selective amplification of the 377bp fragment (Figure 1). The MnlI restriction enzyme was used to further digest the 377bp PCR products, yielding 4 DNA fragments of 171, 94, 60 and 36bp in wild type and 5 DNA fragments of 231, 171, 94, 60 and 36bp in mutant types (Figure 2).

Data was analysed using SPSS 25. Shapiro Wilk's test was used to check data normality. Mean and median values were reported for quantitative variables, while percentages and frequencies were reported for qualitative data. Independent t-test and Mann-Whitney U test were applied on quantitative data that had normal

and non-normally distribution, respectively. Chi-square test was used to compare qualitative data between two genotypes. Pearson correlation test was used to observe correlation between normally distributed quantitative parameters, and Spearman's correlation was used for non-normally distributed quantitative parameters. P<0.05 was considered significant.

Results

Of the 39 patients, 15(38.5%) were males and 24(61.5%) were females. The overall median age was 14 (23) years. There were 29(74.4%) patients with a thalassemia family history, and 22(56.4%) had a positive family history of transfusion related to thalassemia, while no patient had a family history of iron therapy. The median HbA, HbA2 and HbF levels were 72.2 (65.2-79.1) %, 26.6 (19.1-34.0) % and 0.9 (-0.8-2.6) %, respectively. After molecular investigation, HbAE mutation was found in 23(59%) patients, while wild type HbAA genotype was found in 16(41%). The heterozygous HbE mutation was present in 23(59%) patients. Median age, RBC count, haematocrit (Hct), mean corpuscular Hb concentration (MCHC), and HbF did not differ significantly between the HbAA and HbAE genotypes (p≥0.05). Median HbA and HbA_2 levels of patients with HbAA and HbAE genotypes showed a significant difference (p≤0.05). Mean Hb, MCV, MCH and platelet count of HbAA and HbAE genotypes did not differ significantly (p≥0.05).

Even though females were in majority in both genotype

Table-1: Comparison of demographic, clinical and haematological parameters between haemoglobin (Hb) wild type (AA) and mutant type (AE) genotypes.

Study Variables	Category	Enrolled Study Subjects (n = 39)	Genotypes		*p*-value
Demographic and Clinical Features			**Hb AA (Wild type) (n =16)**	**Hb AE (Mutant type) (n = 23)**	
Gender	Male	15 (38.5%)	06 (40%)	09 (41.7%)	0.918[b]
	Female	24 (61.5%)	10 (60%)	14 (58.3%)	
Age (Years)		14 (23)	17.0 (26)	12.0 (17)	0.508 [d]
Transfusion History	Yes	04 (10.3%)	Nil (0.0%)	04 (10.3%)	0.129[a]
	No	35 (89.7%)	16 (100%)	19 (89.7%)	
Family Transfusion History	Yes	22 (56.4%)	05 (31.2%)	17 (73.9%)	0.008 [b]
	No	17 (43.6%)	11 (68.8%)	06 (26.1%)	
Family Thalassemia History	Yes	29 (74.4%)	09 (56.2%)	20 (87%)	0.059[a]
	No	10 (25.6%)	07 (43.8%)	03 (13%)	
Haematological Parameters					
RBC (x10^6/µl)		4.90 (4.35-5.45)	4.92 (4.54-5.30)	4.84 (4.30-5.37)	0.424 [d]
Hb (g/dl)		9.1±2.9	9.8 ± 2.1	8.6 ± 3.4	0.183[c]
Hct (%)		33.7 (29.5-37.8)	34.6 (31.2-37.9)	32.3 (25.0-39.6)	0.103 [d]
MCV (fl)		71.1±9.6	72.50 ± 10.46	70.06 ± 9.09	0.617 [c]
MCH (pg)		20.0±3.9	20.32 ± 3.90	19.83 ± 4.05	0.704 [c]
MCHC (g/dl)		28.7 (26.2-31.2)	28.2 (26.2-30.2)	29.3 (26.2-32.3)	0.521 [d]
Platelets (x10^3/µl)		335.1±150.5	360.0 ± 109.04	317.83 ±173.99	0.359 [c]
HbA (%)		72.2 (65.2-79.1)	74.0 (67.0-81.0)	70.6 (47.4-93.8)	0.027 [d]
HbA_2 (%)		26.6 (19.1-34.0)	24.7 (19.2-30.2)	29.1 (22.1-36.0)	0.022 [d]
HbF (%)		0.9 (-0.8-2.6)	1.35 (0.32-2.37)	0.80 (-8.85-9.65)	0.83[d]

RBC: Red blood cell, Hct: Haematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular haemoglobin, MCHC: Mean corpuscular haemoglobin concentration.
a: p value is generated by Fisher Exact Test, b: p value is generated by chi-square test, c: p value is generated by Independent t test, d: p value is generated by Mann Whitney U test. P≤0.05 was considered significant.

groups, gender difference was not statistically significant (p=0.918). Family history of transfusion was significant between the genotype groups(p=0.008) (Table-1).

Discussion

Haemoglobinopathies have no specific medical treatment, and the WHO has advocated a lifestyle change programme for prevention and control, which includes public education, screening for prenatal detection and asymptomatic carriers, and genetic consultation.[15,16] In a prior investigation in Pakistan, there were 7(0.22%) HbE carriers and 1 case of compound heterozygous (HbE/Beta thalassemia). On HPLC, they all displayed a microcytic hypochromic blood picture and a considerable rise in HbA2 (>8%).[17] The current study was planned to identify cases of hidden HbE while resolving the diagnostic challenge haematologists face when employing HPLC to detect HbE because HPLC retention times for HbE and HbA_2 are similar and they co-elute in the same window. Therefore, to deal with issues like these, molecular techniques are employed. However, there are just a few large settings available in this regard, and laboratories do not generally carry out such investigations.[12, 18]

The present study was conducted on 39 subjects with suspected haemoglobinopathies. Out of these, 24(61.5%) were females and 15(38.5%) were males. This gender distribution was comparable to another Pakistani study that showed a total of 57.5% females and 42.5% males.[3] Similar results were seen in a study conducted in India which showed 53.48% females and 46.52% males.[19] Higher rates of aberrant Hb patterns were observed in female study participants. This can be explained by the fact that pregnant females frequently have a Hb measuring test performed to assess their physical condition and anaemia during the first perinatal visit. In a typical prenatal exam, the HPLC of pregnant female is also included. These contributions predict Hb abnormality.[20]

According to the data collected, 29/39 (74.4%) subjects had diagnosed thalassemia patients among their first and second degree relatives. The results of the current investigation mostly concurred with those of a prior study carried out in Pakistan, which demonstrated that family marriages were a major factor in the high carrier incidence among close relatives. Consanguineous marriages (which make up 70% of marriages in Pakistan) and erroneous information are the main causes of the country's high carrier ratio.[21-23]

In the current study, the mean MCV was 71.06fl, which was consistent with the results of Matos et al.[24] The median RBC count was 4.90 (1.06) $x10^6$/µl. A similar observation was made by another Pakistani study.[3] The mean Hb concentration was 9.1 g/dl, which was lower than a study of Thai blood donors in which 2 donors with homozygous HbE had normal Hb levels (12.7 and 13. g/dL) with very low MCV (61.6 and 67.6 fL) and MCH (19.7 and 22.0 pg).[25]

The present study showed that prevalent genotypes were wild type AA and mutant type AE. The presence of AE was 59% which was comparable to 49% reported earlier.[26]

The haematological parameters were compared between AA and AE groups, which showed that the median RBC count of the AA genotype was 4.92 (4.54-5.30) $x10^6$/µl and it was 4.84 (4.30-5.37) $x10^6$/µl for AE genotype. Mean Hb was 9.8 ±2.13 g/dl for AA, while it was 8.6 ± 3.36 g/dl for AE. The mean MCH was 20.32 ± 3.90pg for AA, while it was 19.83 ± 4.05pg for AE. The difference was statistically non-significant except for HbA and HbA2 distribution. Similar results were reported in another study.[27]

In the present study, the median HbA2 was 24.7 (19.2-30.2) % for AA and it was 29.1 (22.1-36.0) % for AE (p=0.022). HbA2 and HbE had almost the same retention time (3.48 min), which could be one of the causes of increased HbA2 percentage. Previous studies support the findings of increased HbA_2 in patients with HbE disease.[26-27]

The current study has limitations as the sample size was not large enough to accurately reflect HbE prevalence in the community. Besides, owing to time and financial restrictions, molecular confirmation of beta thalassemia could not be done.

Despite the limitations, the current study used PCR amplification and RFLP technique for the first time in the Punjab province of Pakistan on patients with high HbA2 levels.

Conclusion

Frequently missed/undiagnosed cases of HbE that co-elute with HbA2 in the same HPLC window were detected among those with HbA_2 ≥7%.

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