ORIGINAL RESEARCH

Haemoglobin among patients with Sickle Cell Disease with high performance liquid chromatography

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ABSTRACT

Background: Measurement of HbA2 in sickle cell illness is critical for distinguishing between sickle cell anaemia (HbSS) and Hb S/0-thalassemia. The goal of the current research is of assessing the magnitude of HbA2 among sufferers diagnosed with SC hemoglobinopathy and also HbSS, with or without associated alpha thalassemia, with the help of High-Performance Liquid Chromatography (HPLC).

Methods: In the current retrospective study, 242 children belonging to the ages of two to six years old who had HbSS or HbSC diagnosis were involved. The haemoglobin was tested with the help of HPLC. Polymerase chain reaction (PCR) was instrumentalised for detecting alpha thalassemia. Patients were categorised into three groups: homozygous (3.7/3.7), heterozygous (3.7/), and homozygous wild-type (3.7/3.7). The mean HbA2 values with alpha thalassemia were compared using variance analyses.

Results: The HbA2 concentrations in the HbSS group (n = 135) were 3.68 0.65 percent on average (standard deviation). Individuals with HbSS who were heterozygous (n = 28) or homozygous (n = 3) for alpha thalassemia had mean values of 3.98 and 4.73 respectively. The mean HbA2 of all HbSC sufferers (n = 107) was 4.01 0.507, with 4.29 0.41 percent heterozygous for alpha thalassemia (n = 23) and 4.91 0.22 percent homozygous for alpha thalassemia (n = 7) respectively. HbA2 values were above 3.5 percent in all patients homozygous for alpha thalassemia. HbA2 values greater than 5.2 percent were seen in sufferers with HbSS and HbSC, regardless of the presence of alpha thalassemia.

Conclusion: HbA2 levels are higher in patients with HbS or HbC, and alpha thalassemia genotypes have a direct impact.

Keywords: Sickle Cell Disease, Hemoglobinopathy, Fetal haemoglobin, Haemoglobin A2, high-performance liquid chromatography

INTRODUCTION

Sickle cell disease (SCD), as known as Sickle Cell Anemia is amongst world's most frequent hereditary diseases. There is a wide range of clinical symptoms among sickle cell anemia patients, and multiple factors are linked to the various appearances (Weatherall [1]). Some variables like laboratory, clinical and genetic factors, are well-known, whereas psychosocial

and dietary factors, have received less attention (Nogueira, et al [2]). The significance of the hemoglobinopathy phenotype is well defined among genetic variables, with those with Hb S/0-thalassemia and those doubly heterozygous for sickle cell anaemia having a more severe clinical profile (Rakyan, et al [3]; Thein [4]). Carriers of HbSC with HbS/+- thalassemia instead manifest better outcomes, making accurate treatments of these syndromes critical for appropriate clinical and therapeutic care of sufferers (Greene, et al [5]).

Quantification of HbA2, as well as a complete blood count (CBC), anamnesis, aid in differential diagnosis of HbSS and Hb S/0-thalassemia in SCD (Thein [4]). The best method for measuring HbA2 properly has become a much debatable topic in medical literature, and HPLC has long been considered a choice. Among healthy people without thalassemia, the reference value for HbA2 is usually between 2.0 and 3.3 percent (Head, et al [6]).

The determination of different Hb fractions is significant not only for prognosis but also for diagnosing various hemoglobinopathies. Electrophoresis, alkali denaturation, and radial immune diffusion are a few of the methods which are instrumentalised for quantifying diverse Hb. For the first screening of haemoglobinopathies and quantifying haemoglobin fractions such as HbA, HbA2, and HbF 7, HPLC has been frequently used (Clarke, et al [7]); Greene, et al [8]; Paleari, et al [9]). Given the large number of SCD patients in this area of the country, the scarcity of research on this component of the disease, and the availability of the most up-to-date HPLC equipment, it was thought worthwhile to conduct this study to quantify main Hb fractions (Paleari, et al [9]).

Suh, et al [10] reported in 1996 that HbA2 levels determined by HPLC elevated dramatically amongst samples who have HbS. This suggests that HbSS sufferers might have been misdiagnosed with Hb S/0-thalassemia. They also indicated that the presence of smaller HbS components that co-eluted with HbA2 could explain increases in HbA2. Shokrani et al. [11] reported in 2000 that samples of blood from HbSS or HbSC patients and persons with HbAS with Hb values of 5.9% and HbA2 values of 5.2 percent as measured by HPLC can be regarded normal.

In 2004, Head, et al [12] validated Shokrani, et al findings explaining that the inaccurately elevated HbA2 levels had been caused by the existence of HbS which underwent post-translational modifications. Hence, it had similar retention time as HbA2. They also discovered that the uncertainty level is proportional to the concentration of HbS, which is elevated among HbSS patient samples than in HbAS patient samples (Head, et al [12]). They also discovered that the percentages of HbA2 among the HbAS sufferers with AT is elevated in contrast with those without AT. This is as a consequence of the delta chains' higher affinity for alpha chains than the S chains (Zurbriggen, et al [13]). More recently, mass spectrometry investigations have suggested that higher HbA2 values in HbS samples may be due to the elution of smaller Hb components generated by a S chain connected with an alpha-globin chain that was carbamylated after translation (Higgins, et al [14]). The current study aims to verify HbA2 values which are quantified by HPLC in samples of sufferers with HbC and HbS in the absence or presence of the 2-thalassemia 3.7 kb omission, as both hemoglobinopathies might be prevalent amongst the same patients.

METHODOLOGY

SAMPLE

From January 2018 to December 2020, this research was undertaken in the Department of Medicine, VSS Institute of Medical Sciences and Research, Burla, and the Gupta Diagnostic and Research Centre, Burla. This study involved 287 patients who went to the Medicine OPD and Gupta Diagnostic Centre for SCD screening.

LABORATORY TECHNIQUES

Following a thorough clinical examination, blood was drawn for a thorough haematological analysis in 3.0 mL tubes which contained 3.6mg K2-ethylenediaminetetraacetic acid (EDTA) in the form of an anticoagulation agent. In all cases, Hb electrophoresis and a sickling test on an agar gel were performed. HPLC was employed in each case for hemoglobinopathy screening and measurement of main haemoglobin components. It was carried out using the Bio-Rad variant Hemoglobin testing system and the variant $\alpha^{3.7}$-thalassemia short programme from Bio-Rad Labs. A vacuum blood collection tube containing EDTA as an anticoagulant was instrumentalised for collecting whole blood for this study. 5 litres of blood were pipetted into a 1.5 ml sample vial. 1 mL hemolytic reagent was added to it. Inversion was utilised for covering and mixing each sample vial. The programme was started after the sample vial was placed in the variation sample tray. When a batch of 15 samples was ready, the blood was taken and stored at –100C for the test. At the beginning and conclusion of each group of patient specimens, a set of normal (Hb F 1-2 percent, HbA2 1.8-3.2 percent) and pathological (Hb F 5-10 percent, HbA2 6 percent) controls were conducted. Each Hb fraction's percentage is determined and examined depending on the chromatographic analysis.

STATISTICAL ANALYSIS

Using central tendency analysis and dispersion, HbA2 concentrations were reported as per the type of sickle cell illness (HbSC or HbSS) and AT status. For normal distribution analysis, the Kolmogorov–Smirnov test was used. The impacts of the interaction and confounding factors between sickle cell disease types and presence of AT on HbA2 levels was investigated using a one-way ANOVA. A two-way ANOVA was instrumentalised for investigating the influence of the interaction and the confounding factors. To identify circumstances where equality of variance was proven, post hoc analysis was performed using the Games–Howell test and Tukey's Honestly Significant Difference (HSD) test. Because the group with homozygous mutations was small, statistical significance was defined at a p-value of 0.01. The chi-squared tendency test was instrumentalised for assessing these groups in relation to the AT data. EPI INFO for Windows and the Statistical Package for the Social Sciences were instrumentalised for analysing all data.

RESULTS

The selected participants of this study were screened for hemoglobinopathies while the major fractions of haemoglobin were assessed by HPLC. The gender and age distribution have been provided in Table 1 below. The majority of the participants belonged to the age range of 21-30, and the male to female ratio was 1.4:1.

Sickle cell disease	**$\alpha^{3.7}$-Thalassemia**						**p-value**
	αα/ αα		- α/αα		- α/-α		
	n	%	n	%	n	%	0.233
HbSS	104	77.0	28	20.7	3	2.2	
HbSC	77	72.0	23	21.5	7	6.5	
Total	181	74.8	51	21.0	10	4.1	
HbSS; sickle cell anemia; HbSC; SC hemoglobinopathy							

Table 1: Distribution of $\alpha^{3.7}$- thalassemia in 242 children with sickle cell anemia

Among the HbSS patients, the mean HbA2 level with heterozygous AT (n = 28) was 3.98 percent, without AT (n = 135) was 3.68 percent and with homozygous AT (n = 3) was 4.73 percent. In all HbSC patients, HbA2 was 4.01 percent; with heterozygous AT (n = 23) was 4.29 percent, and with homozygous AT (n = 7) was 4.91 percent. In terms of HbSS or HbSC and mean HbA2 levels, sufferers with HbSC had significantly higher overall values than

patients with HbSS (p-value 0.001), as indicated in the table below. In case data was stratified according to homozygous, heterozygous and wild type, significant differences emerged when samples without AT were contrasted.

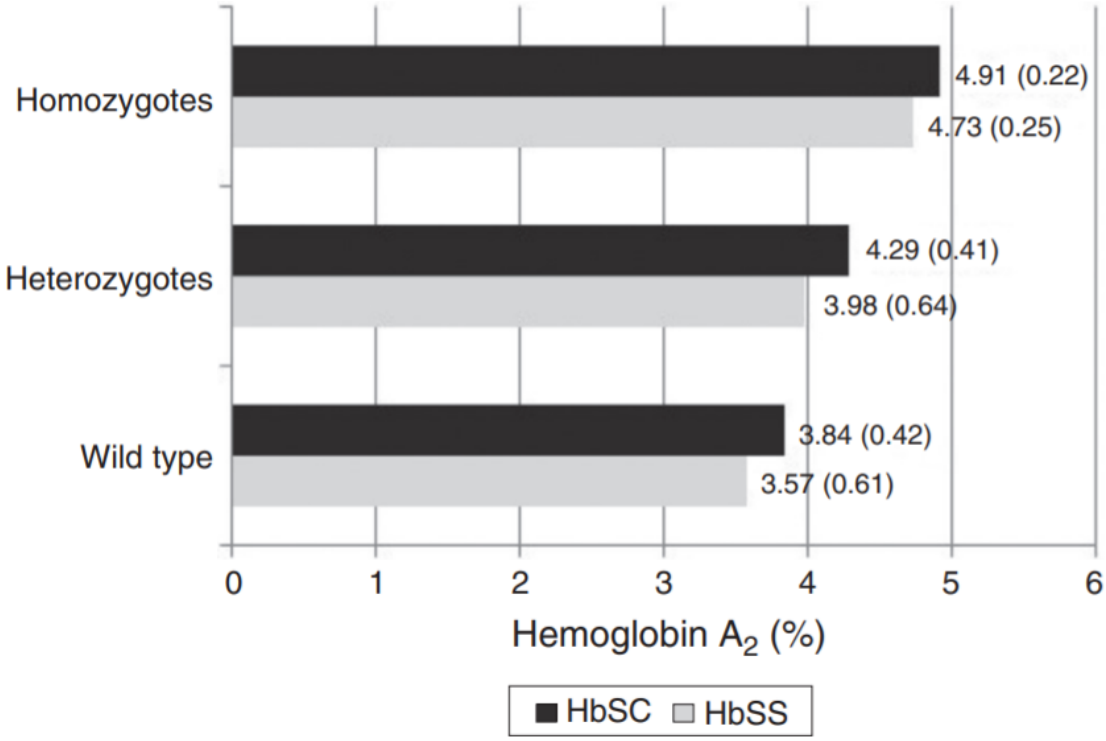

Figure 1. HbA_2 values according to 3.7-thalassemia genotype and sickle cell type

$\alpha^{3.7}$-Thalassemia	Mean	SD	Minimum-Maximum	p-value
αα/αα	3.68	0.56	1.50-5.20	0.001
HbSS	3.57	0.61	1.50-5.20	
HbSC	3.84	0.42	2.90-4.80	
-α/αα	4.12	0.57	2.30-4.90	0.074
HbSS	3.98	0.64	2.30-4.90	
HbSC	4.29	0.41	3.30-4.90	
-α/-α	4.86	0.23	4.50-5.20	0.267
HbSS	4.73	0.25	4.50-5.00	
HbSC	4.91	0.22	4.60-5.20	
Total	3.82	0.62	1.50-5.20	<0.001
HbSS	3.68	0.65	1.50-5.20	
HbSC	4.01	0.51	2.90-5.20	

Table 2. Mean, SD, maximum and minimum values of HbA_2 according to presence of $\alpha^{3.7}$-Thalassemia

Following that the difference between the average HbA2 values for HbSC and HbSS was substantial, the relationship between AT genotype and sickle cell anemia type was investigated. There was no significant interaction or influence of the type of hemoglobinopathy on the mean HbA2 levels. Only the AT genotype had an effect on the variation of mean HbA2 values. The post hoc analysis had revealed significant differences between three AT genotype groups, with gradual increase in HbA2 levels.

DISCUSSION

$\alpha^{3.7}$-thalassemia and HbSS are the hemoglobinopathies that have the largest influence on mortality and morbidity (Ondei, et al [15]). Owing to the fact that it is triggered by presence of an aberrant Hb variation – HbS – sickle cell anemia should be considered both a quantitative and qualitative genetic illness (Amorim, et al [16]). The most prevalent genotype is homozygozity for HbS. Compound heterozygous states of HbS with HbC or $\alpha^{3.7}$-thalassemia variations are additional causative genotypes.

α_2-Thalassemia was developed in 23.4 percent of children with sickle cell illness, a finding that matches prior researches by Dode, et al [17] also cited in World Health Organisation [18]. As stated by Suh, et al. [10] and Ondei, et al. [15], presence of AT influences upon the value of HbA2 as measured by HPLC. Significant distinctions in mean amount of HbA2 had been determined in this research, with an elevation in Hb fraction between wild type, heterozygous, and homozygous (Kalleas, et al [19]).

During the recent years, several research have attempted to examine various approaches so as to find processes where the presence of aberrant Hbs has least influence on measurement of HbA2 (Anagnostopoulos, et al [20]). It allows for a more accurate diagnosis (Anagnostopoulos, et al [20]). However, investigations looking into how the presence of these AT and Hbs might lead to misleading HbA2 levels are limited. Even recent studies, such as Greene, et al [8] appear to concur with Suh, et al [10] in attributing apparent rise in HbA2 as measured by HPLC among people with Hb structural variations to the existence of glycosylated fractions of anomalous Hb that co-elute with HbA2. During evaluation of the impacts of Hb variants on HbA2 measurement, the presence of AT had the greatest impact. Head, et al [12] defended that the increased HbA2 levels manifested the association of sickle cell disease with AT. This can be explained by the fact that fewer alpha chains are produced, allowing these positively charged molecules to combine with other chains for which they have a strong affinity, namely the delta chains.

The study community has a relatively low prevalence of α_2-Thalassemia and a careful haematological examination of the parents revealed normal erythrograms and the presence of HbS and HbA, indicating heterozygosity for HbS (Fonseca, et al [21]). We also believe that the sufferers should be diagnosed with sickle cell anaemia rather than HbS/0 α_2-Thalassemia (Couto, et al [26]). Despite the fact that we did not perform molecular analyses to conclusively rule out α_2-Thalassemia. We recommend that in cases where a possible diagnosis of HbS/0 α_2-Thalassemia is suspected, the Hb profile be conducted by HPLC instead, or that if it is utilised, it be accompanied by a second confirming test. In addition, if there are any remaining doubts, a molecular investigation should be performed to find deletions or other mutations, which will confirm the clinical diagnosis and lead genetic counselling.

CONCLUSION

In conclusion, the current investigation revealed that SCD is the most common form of hemoglobinopathy in this region of the country, both homozygous and heterozygous. HbF concentrations are observed to be higher in SCA patients, and it is thought to be a key element in protecting sufferers from problems. Our conclusion is that when the levels of HbA2 containing HbS and/or HbC are examined by HPLC, they can be exaggerated, especially in the presence of AT. Another conclusion is that reference values of up to 3.5 percent should not be employed in these instances. We emphasise the significance of multi-center studies for establishing patterns, the exigency for individual case evaluations to reach a differential diagnosis between SCD, especially in areas with a high prevalence of the various types of hemoglobinopathies, and the use of molecular biology studies to clear any doubts.

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