Predicting the severity of sickle cell disease using hematological, biochemical, and cellular parameters

Running title: Lab parameters and sickle cell severity

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Abstract

Background: Sickle cell disease, a hemoglobinopathy caused by a point mutation, has a heterogeneous clinical course. The level of Hb F within erythrocytes is believed to be the most important parameter for the severity of disease. The aim of this study is to investigate whether Hb F level, F cell count, and sickle cell percentage after in vitro induction of sickling can predict the severity of the disease.

Methods: All necessary data were collected from clinical history, biochemistry, and pathology lab tests. This is a cross-sectional study with 31 participants. Statistical analyses were done by using the correlation coefficient and chi-square test to find a significant difference between two variables. Statistical analysis was performed using MedCalc software.

Results: The majority of patients fell into the mild severity score category; with a lack of severe disease phenotypes. The number of painful episodes, hospitalizations, and cumulative disease severity scores were associated with high levels of LDH and indirect bilirubin. However, none of the clinical disease severity parameters or the overall cumulative disease severity score was associated with Hb F level, F cell count, or the percentage of sickled cells after in vitro induction of sickling. However, a high percentage of F cells were associated with high MCV, MCH, and MCHC and low RDW, LDH, and indirect bilirubin.

Conclusion: This sickle cell disease severity is related to susceptibility of RBCs to hemolysis as indicated by s. LDH and indirect bilirubin levels. However, extent of hemolysis may be dependent on multiple factors rather than F cell count or Hb F level only.

Key words: Sickle cell disease, F cell count, Hb F, LDH, Sickle cell percentage

Introduction

Sickle cell disease is a chronic and debilitating genetic condition resulting from a single point mutation in the β-globin gene of hemoglobin. This point mutation will make erythrocytes prone to acquire sickle shape under hypoxic condition and premature hemolysis.

In India, sickle cell disease is one of the most frequently encountered hemoglobinopathies, specifically in central, southern and north–eastern parts of India. In Gujarat, the sickle cell gene has an overall prevalence of about 6.5%, rising to nearly 11% in tribal populations (1).

Sickled erythrocytes are less flexible and sticky. While travelling through peripheral circulation they cause vessel obstruction, stasis and hyper-viscosity which leads to haemorrhages, infarctions and ischemic necrosis of tissue/organs throughout the body causing complications like vaso-occlusive crisis and associated stroke, splenic sequestration crisis, jaundice, leg ulcers, priapism and painful episodes.

Despite the uniform underlying point mutation, clinical severity varies markedly among patients from different geographical areas and communities. Sickle cell disease is of mild phenotype in Indian population overall, but there is wide variation of disease severity in individual patient (2).

Hb F and Hb S content within erythrocytes is thought to be the most important factor determining severity of disease by determining polymerization and sickling. However, Relation between Hb F percentage and clinical severity of the sickle cell disease is variable. It is postulated that a heterogeneous distribution of Hb F within erythrocytes could be reason and Hb F content in individual erythrocyte could be a critical determinant of the Hb F effect (3,4).

Hb F as percentage of total Hemoglobin can be detected by HPLC or capillary electrophoresis which gives the percentage of HbF in a hemolysate but not in individual Erythrocytes. There are also immunological and chemical methods which estimate the percentage of Erythrocytes containing increased amount of Hb F compared to normal adult erythrocytes, named F cells (3). It is not possible to reliably estimate Hb F in individual erythrocytes but Nicolas Hebert et al have attempted the same in their study (5). If this parameter can be obtained, theoretically, it is supposed to be most ideal parameter to demonstrate protective effect of Hb F on severity of disease.

Our study has ttried to explore relation of disease severity of paediatric patients on hydroxyurea therapy with Hb F% by HPLC as well as flowcytometric assessment of F cell count as percentage of total erythrocyte count. F cells being cell with higher Hb F levels are supposed to be protective against sickling. In line with this hypothesis, we further investigated the association between the percentage of sickled cells following induced in lab setting and the clinical severity of the disease.

Additionally, we evaluated various hematology and biochemical parameters, such as LDH and bilirubin levels, to identify the parameter that best correlates with the severity of sickle cell disease in patients on hydroxyurea.

Methods

A one-year time bound cross sectional study was conducted in the western part of India. A total of 31 outdoor pediatric patients (less than 13 years of age) with sickle cell disease were included in study after taking consent of parent or guardian. Patient, having blood transfusion in last 3 months, were excluded from study as recent transfusion may affect some of the variable under study. Blood samples were obtained for various laboratory tests including F cell count.

Demographic, clinical and laboratory data were collected from the Pediatrics, Pathology and Biochemistry departments. F cell count data was obtained from private laboratory as samples were outsourced.

Structured questionnaires were employed to obtain the current clinical profile and past medical history, including details on the age at initial diagnosis. Information was obtained about degree of splenic and hepatic enlargement, frequency of complication in past 12 months, and lifetime incidence of complications for each patient. Past medical records were reviewed in case of ambiguity in information.

Scoring of severity of sickle cell disease was performed was identical to Samuel Ademola et al (6). Six parameters were used to assess the patient's present state, their clinical status during the past 12 months, and the severity of lifetime complications. Each item was scored based on its frequency and/or severity, using a scale from 1 to 5. The total score (0-30) was computed for each child, after which disease severity was categorized as mild (<5), moderate (6–17), or severe (>17).

Complete blood count, serum lactate dehydrogenase, and bilirubin levels were assessed using standard laboratory procedures during the outpatient department visit.

A 5-part automated cell counter (HORIBA Pentra XLR) was used to perform the complete blood count. Serum LDH done by fully automated ERBA XL-640 analyser. Bilirubin done by semi-automated Microlab ARX 50-V analyser. Then the blood samples were subjected further for, High Performance Liquid Chromatography (HPLC) on Bio-rad VARIANT II HPLC device. F cell count was sent to a private lab and was performed on DeFLEX Flow-cytometer using FITC conjugated mouse monoclonal antibody against fetal hemoglobin.

In-vitro induction of red cell sickling was performed as per method similar to that described by Oyenike MA et al (7). Solution of 20ul 2% sodium metabisulfite was added to 20µl of washed erythrocytes, mixed well, and sealed with liquid paraffin to exclude air and maintain hypoxia. Results were documented after 24 hours. Counts of sickled and total erythrocytes (normal and abnormal) were obtained from five fields randomly selected across the slide. Results obtained as percentage of sickled erythrocyte.

Statistical analysis was performed using correlation coefficients to examine relationships between variables and chi-square tests to assess the significance of differences between groups. MedCalc software was used to conduct all statistical analyses.

Results

The study population consisted of 31pediatric patients, with5 (16%)aged ≤5 years, 15(48%)aged6-10-years, and 11 (36%)aged11-13 years. The average age was 9years. The study population included 22(71%) male children and 9 (29%) female children. The mean age at first time presentation of the patients was 4.7 year.

A large proportion of the study population comprised follow-up cases of sickle cell disease, and the majority reported no current symptoms. Others present with pallor, pain in limbs or abdomen, fever, cough and hematuria.

Table 1 summarizes the clinical characteristics of the patients with sickle cell disease in present study. 32% of the patients had one painful episode in one year with only 19% subjects had more than 3 painful episodes in one year. 52% of the patients required no blood transfusion in one year. There were only 13% of subjects who required 2 or 3 blood transfusions in one year. 52% of the patients required one-time hospitalization in one year with only 1 subject requiring more than 3 times in the last year.94% of patients had liver enlargement less than 2 cm with only 1 subject having 2-5 cm liver

enlargement and 1 subject having more than 5 cm liver enlargement.84% of patients had spleen enlargement less than 5 cm with only 1 subject having 5-10 cm spleen enlargement. Only 1 subject had past history of cerebro-vascular event and 1 subject had chronic leg ulcer. Around 19(62%) have moderate anemia (7 to 10gm of Hb/dl) category, 8(25%) subjects in the mild anemia category and 4(13%) subjects in the severe anemia category.

On HPLC testing, patients have meant of Hb F and Hb S were 19.5 % ±5.4 and 72.3 % ± 5.8 respectively.

Table 2 shows the results of correlation between laboratory parameters and clinical parameters. Hb F showed no significant correlation with any clinical parameter. There is no significant correlation between in vitro sickle cell percentage and any clinical parameter.

Painful episodes were significantly correlated with MCHC, LDH, total bilirubin, and indirect bilirubin. Painful episodes and MCHC had an inverse relationship while having a direct relation with LDH, total bilirubin and indirect bilirubin.

The number of hospitalizations was significantly correlated with LDH, total bilirubin, and indirect bilirubin. The number of blood transfusions was significantly correlated with hemoglobin, hematocrit, and LDH. Finally, laboratory parameters which significantly correlated with disease severity score were LDH, total bilirubin and indirect bilirubin. Notable negative finding is F cell count, invitro sickling percentage or Hb F level does not correlate with any of the clinical severity parameter. Serum LDH and Indirect bilirubin level is found to be correlating with disease severity while considering most of the clinical severity parameters.

Table 3 show correlation of disease severity scores with various laboratory parameters. Severity scoring was performed for 31 subjects, of whom 25 (80%) had mild disease and 6 (20%) had moderate disease. No subjects were found with severe category. None of the subjects met the criteria for the severe category.

Table 3 presents a comparison of various hemogram parameters across the different categories of disease severity. LDH, total bilirubin, and indirect bilirubin showed statistically significant differences ($p = 0.004$, 0.007, and 0.018, respectively), while MCV was close to significance ($p=0.056$).

Figure 1 illustrates the correlation between F-cell count and various laboratory parameters. MCV, MCH, MCHC, RDW, LDH, and direct bilirubin demonstrated significant positive correlations with F-cell count. F cell Count was directly related to MCV, MCH, and MCHC; and inversely related to RDW, LDH and total bilirubin.

Discussion

Our findings indicate that certain parameters demonstrated significant correlations with disease severity, while others, conventionally regarded as severity markers in sickle cell disease, did not correlate with severity among patients undergoing hydroxyurea therapy. In this study, the majority of patients were asymptomatic or exhibited only mild symptoms, likely because they were follow-up cases who were clinically stable and managed on an outpatient basis.

Majority of patients are in mild severity score category. None of the patient falls into severe as per disease severity score. There was an average of moderate anemia. There was a relatively less number of painful episodes overall in all patients and painful episodes are associated with a decrease in MCHC, increase in LDH, total bilirubin and indirect bilirubin.

The present study showed number of hospitalizations associated with high levels of LDH, total bilirubin and indirect bilirubin. Only a few patients had significant (score 2/3) liver or spleen enlargement.

There were no patients with acute chest syndrome, pneumococcal meningitis, avascular necrosis, gall stones, osteomyelitis, or priapism in present study.

In overall cumulative disease severity score was directly associated with LDH, total bilirubin and indirect bilirubin, a high score was associated with the high level of LDH, total bilirubin and indirect bilirubin. This observation can be attributed to the heightened vulnerability of RBCs to splenic destruction in more severe disease, given that serum LDH and bilirubin levels serve as indicators of the degree of hemolysis.

However, none of the clinical disease severity parameters and overall cumulative disease severity score was associated with hematology parameters like Hb F level by HPLC which is commonly used to monitor patient on hydroxyurea. Other tested parameters, such as F-cell count and the percentage of sickled cells following in vitro induction, also failed to demonstrate a significant correlation. These findings indicate that HbF levels or F-cell counts are not related to disease severity in patients with sickle cell anemia. However, as shown in Figure 1, higher F-cell counts are positively associated with increased MCV, MCH, and MCHC, while demonstrating an inverse relationship with LDH levels. No significant correlations were observed with other hematological parameters. Okocha E, et al. found a significant positive correlation of disease severity with MCHC, MCV and WBC and a negative correlation of disease severity with Hb and PCV (8). Powar DR et al observed no correlation between severity of sickle cell disease and Hb F levels & RBCs indices (9).

The result of the present study does not agree with the general opinion that a high level of Hb F has a better effect on the severity of sickle cell anemia or at least there is no effect in mild and moderate severity cases. Similar to the result drawn by Powar DR et al (9)., Ozsoylu et al (10), Serjeant et al (11) Although these studies had the lower mean value of Hb F than our study mean (19.5%). The strongest evidence supporting the beneficial effect of elevated Hb-F levels on the severity of sickle cell disease comes from studies of Arabian patients with sickle cell anemia (11–15). In the study by Perrine et al. (15), Arabian SS cases demonstrated marked differences in their clinical presentation. In their study, 75% of the Arabian SS patients tend to be mild in its manifestations. In our study also had relatively mild symptoms but after hydroxyurea therapy.

Our study demonstrated limited variability in disease severity, with most patients falling into the mild score category. In contrast, Alabid T et al. reported a wider variability in severity patterns among Sudanese patients with sickle cell disease, using a scoring system based on simple clinicopathological parameters. In their study, most of the patients were in the moderate and severe score category and only 21% of patients were in the mild score category which is opposite to our study (16). This further supports the notion that disease severity is influenced by multiple genetic factors, which vary across geographical regions, as well as by environmental, socio-economic, and treatment-related differences. The study supports belief that Indian sickle cell disease patients are well responder and have relatively mild disease on hydroxyurea treatment. Adegoke SA et al. reported that sickle cell disease in children exhibits wide clinical variability in severity. In their study, 33.9% of patients had mild disease, 55.7% had moderate disease, and 10.4% had severe disease based on their severity scoring system. They found hematological parameters were of higher range in severe disease while Hb F was lower side (17). However different studies use their own disease severity scoring system which can be potential source of variable results.

There was no correlation of sickling in vitro with any of the severity score or laboratory parameter except high percentage of sickled erythrocytes were associated with high level of F cell percentage. This could be explained by the fact that high level of feta

haemoglobin is helpful in preventing polymerization of Erythrocytes but the finding is not associated disease severity.

F cell count does not correlate with any of the clinical parameter as well as cumulative clinical severity score. It is believed that Hb F has protective effect on sickling and that was the basis of protective effect of higher Hb F with mild disease. But patient on hydroxyurea therapy, this Hb F level and F cell count does not correlate with disease severity. So probably other factors and mechanisms of action of hydroxyurea therapy might have been more important rather than increasing Hb F level in erythrocytes. Study with larger sample size with greater variability of clinical severity is needed. Another explanation could be F cell count may not truly reflect number of these F cells protected from sickling as quantity of Hb F within individual erythrocytes and number of F cells which have protective levels of Hb F in them might be more relevant. So, Hb F levels and F cell count is not needed to assess severity of sickle cell anaemia patient on hydroxyurea therapy as per our study results.

This study is limited by the reliance on information recalled by patients or their caregivers, which may introduce recall bias. Additionally, the small sample size—partly due to restrictions during the COVID-19 pandemic—may have reduced the statistical power to detect significant associations. All of the patients were on hydroxyurea therapy that may alter relationship between severity and various parameters. There was no representation of patients with severe disease severity score.

Conclusion

- HbF percentage, F-cell count, and the percentage of sickled cells after in vitro induction are not related to disease severity in patients receiving hydroxyurea therapy. Therefore, these tests are not necessary for assessing disease severity in hydroxyurea-treated sickle cell anemia patients.
- In hydroxyurea-treated sickle cell disease patients, serum LDH, total bilirubin, and indirect bilirubin were the only parameters associated with disease severity, indicating that severity reflects increased RBC vulnerability to hemolysis. Nonetheless, hemolysis appears to depend on several factors, not solely on F-cell count or HbF levels.
- Study need to be repeated on a large sample size to check whether results are applicable to population.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Ethical statement

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Author contributions

Viren L. Vaghasiya: Writing – Review and Editing, Writing – Original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization
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Jitendra G. Nasit: Writing – Review and Editing, Writing – Original draft, Project administration, Validation, Supervision, Formal analysis.
Bhoomika Rupavatiya: Writing – Review and Editing, Writing – Original draft, Project administration, Validation, Data curation, Formal analysis

Data availability statement

Additional data supporting study findings are available from the corresponding author on request.

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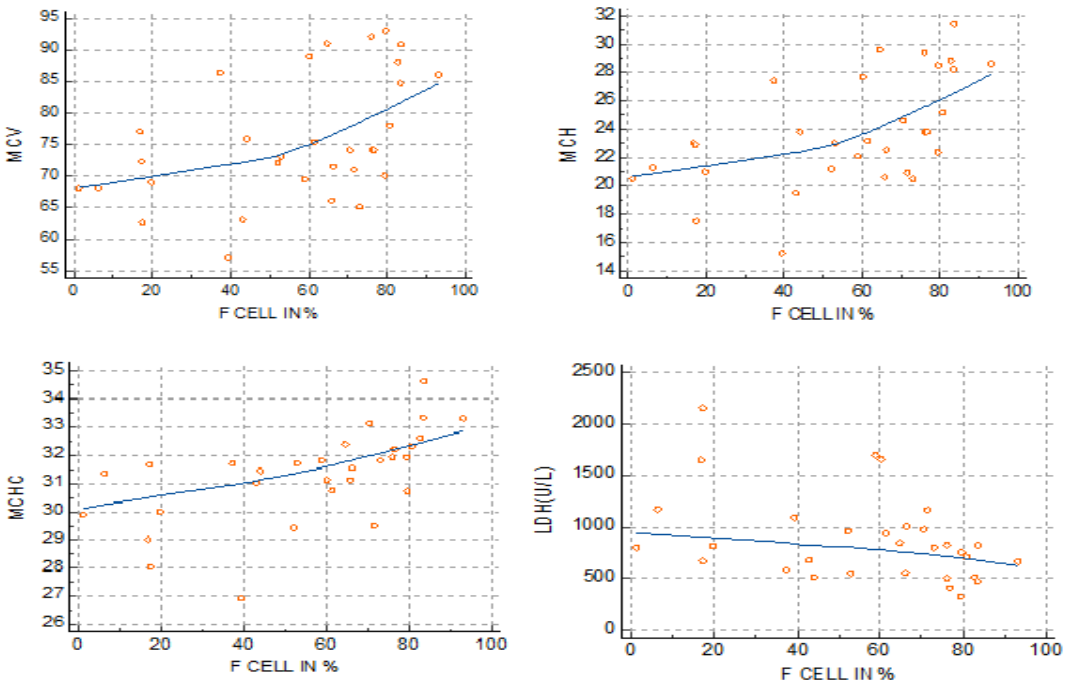

Figure 1. Relation of F cell count to other laboratory parameters in sickle cell disease

Table 1. Clinical characteristics and disease severity score of the study population

	Number of patients					
Severity score	Painful episodes in the last year (%)	Transfusion in last year (%)	Hospitalization in the last year (%)	Liver enlargement (%)	Spleen enlargement (%)	Specific complication (%)
0	7 (23)	16 (52)	10 (32)	29 (94)	26 (84)	29 (94)
1	10 (32)	11 (35)	16 (52)	1 (3)	5 (16)	1 (3)
2	8 (26)	4 (13)	4 (13)	1 (3)	0	0
3	6 (19)	0	1(3)	-	-	0
4	-	-	-	-	-	0
5	-	-	-	-	-	1 (3)

Table 2. Correlation of various clinical severity parameters with laboratory parameters

	Clinical parameter of severity			
Laboratory Parameters	**Painful episodes (P-Value)**	**Hospitalization (P-Value)**	**Transfustion requirement (P-Value)**	**Severity score (P-Value)**
Hemoglobin	0.264	0.266	**0.033**	0.161
HCT	0.434	0.268	**0.030**	0.192
Erythrocyte count	0.977	0.791	0.153	0.828
MCV	0.196	0.173	0.404	0.092
MCH	0.110	0.183	0.317	0.095
MCHC	**0.041**	0.529	0.293	0.256
RDW	0.364	0.765	0.958	0.413
Total WBC count	0.780	0.385	0.944	0.429
Platelet count	0.405	0.694	0.821	0.367
HB A2	0.064	0.676	0.907	0.725
HB F	0.887	0.832	0.487	0.940
HB S	0.499	0.811	0.602	0.565
F cell count	0.188	0.908	0.973	0.645
LDH	**0.001**	**0.001**	**0.0002**	**< 0.0001**
Total bilirubin	**0.000**	**0.002**	0.096	**0.000**
Direct bilirubin	0.651	0.470	0.380	0.479
Indirect bilirubin	**0.005**	**0.004**	0.151	**0.001**
Percentage of sickled cells after induction of in vitro sickling	0.715	0.712	0.610	0.889

Table 3. Comparison of various hematological parameters based on disease severity score

Parameter	Mild severity	Moderate severity	P-Value
Hb (g/dL)	8.8 ± 1.7	8.0 ± 2.2	0.311
Erythrocyte (10^6/µL)	3.7 ± 0.8	3.7 ± 0.9	0.312
PCV(%)	28.2 ± 5.0	25.8 ± 6.3	0.97
MCV(fL)	77.3 ± 10.1	68.9 ± 4.3	0.056
MCH(pg)	24.4 ± 3.9	21.3 ± 2.3	0.082
MCHC(g/dL)	31.4 ± 1.6	30.9 ± 1.8	0.574
RDW-CV (%)	18.7 ± 2.96	19.4 ± 3.49	0.586
Total WBC (/µL)	9296 ± 5115	5583 ± 2102	0.095
Platelet (10^3/µL)	327 ± 1.3	284 ± 1.3	0.476
Sickling in vitro (%)	81.4 ± 17.9	79.5 ± 18.8	0.818
HBF (%)	19.85 ± 6.04	18.25 ± 2.63	0.535
HBS (%)	72.35 ± 6.34	72.52 ± 4.49	0.951
LDH (IU/L)	778.32 ± 335.29	1299.83 ± 519.54	0.004
Total bilirubin (mg/dl)	1.548 ± 0.95	2.73 ± 0.67	0.007
Direct bilirubin (mg/dl)	0.88 ± 1.51	0.93 ± 0.28	0.943
Indirect bilirubin (mg/dl)	0.95 ± 0.76	1.80 ± 0.65	0.018
F cell count (%)	55.8 ± 27.0	59.7 ± 21.3	0.749