Plasma Carbonic Anhydrase II Level is Increased in Children with Sickle Cell Anaemia Compared to Healthy Controls

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Abstract

Introduction: Erythrocyte carbonic anhydrase II (CAII) the bicarbonate supplier causes acidification of the red blood cell hence triggering the dissociation of oxygen from oxyhemoglobin. Mutant hemoglobin S favors sickling in this hypoxic state.

Objective: The purpose of this study was to determine the plasma levels of CA II in known cases of sickle cell disease and to compare them with gender matched healthy controls.

Materials and methods: 40 participants were enrolled for the present case-controlled study with age ranging from 6 months to 12 years. After assessment of complete blood count, sickling and hemoglobin electrophoresis, CAII levels were determined by enzyme linked immunosorbent assay (ELISA).

Result: The mean carbonic anhydrase levels were significantly elevated in children who suffered from sickle cell disease compared to healthy controls (p=0.0001). Carbonic anhydrase II mean levels was significantly enhanced in children who suffered from the clinical complications of sickle cell disease compared to healthy controls (p=0.0001).

Conclusion: Plasma CA II levels were significantly increased in children with sickle cell anemia. The mean carbonic anhydrase II levels were elevated in children with clinical complications of sickle cell anemia.

ABBREVIATIONS

CA: Carbonic Anhydrase; Hb: Hemoglobin; ELISA: Enzyme Linked Immunosorbent Assay; SCD: Sickle Cell Disease; EDTA: Ethylene Diamine TetraAcetic Acid; RBC: Red Blood Cell; WBC: White Blood Cell; SD: Standard Deviation; MCV: Mean Cell Volume; HCT: Hematocrits; MCH: Mean Cell Hemoglobin; MCHC: Mean Cell Hemoglobin Concentration; G6PDH: Glucose 6 Phosphate Dehydrogenase; Plts: Platelets

INTRODUCTION

SCD is an umbrella term of group of autosomal recessive genetic disorders characterized by mutation on the β subunit of the globin chain of hemoglobin within the red blood cells [1]. The disease has been associated with an abnormal variant of hemoglobin (HbS) which causes reversible polymerization of hemoglobin and red blood cell sickling on exposure to low oxygen concentration [2]. Polymerization distorts erythrocyte structure into a variety of sickled shape, damaging the erythrocyte membrane and ultimately causing hemolysis with attendant anemia, ischemia, and infarction and organ dysfunction [1]. SCD complications include chronic anemia, acute chest syndrome, stroke, splenic and renal dysfunction, pain crisis and susceptibility to bacterial infections [3].

In erythrocytes, ubiquitous zinc containing metalloenzyme carbonic anhydrase has been known to catalyze the reversible hydration of CO₂ to H₂CO₃. Carbonic acid (CA) dissociates spontaneously into H and HCO₃⁻ ions. Bicarbonate ions are transferred out of the red blood cells by the band 3 in exchange for chloride ions. This leads to the conversion of a weak acid carbonic acid to a strong acid HCl and renders the pH of the red blood cell acidic hence triggering the dissociation of oxygen from oxyhemoglobin [4]. Mutant HbS favors the occurrence of sickling in this hypoxic state [5]. Thus by extension, enhanced CA II expression promotes sickling of SCD status and the related complications. The first evidence about a factor in red blood cell necessary for the sickling process was from the work of [6]. Following this line of argument [7,8] reported a potential role of CA on the sickling process, in vitro mediated by its effect on the oxygen dissociation curve and erythrocyte membrane.

Little variation in the level of CA isoenzymes has been observed with healthy human population; however a wide variation occurs in various disease status [9]. Two isoenzymes...
of CA are widely distributed in the erythrocytes and since the enzyme in blood is contained wholly within the erythrocytes, it is apparent that CA in blood might be abnormal in anemia [10]. Mondrup [11], illustrated the CA isoenzyme B in erythrocyte of subjects with different types of anemia and that the isoenzyme contributes differently in various anemias. Based on this fact that different types of anemia vary in regard to carbonic anhydrase activity [10,12], investigated the blood CA in anemic blood and illustrated high levels of CA in patients with sickle cell disease and leukemia. We considered it desirable to study this matter again using a different technique. The objective of this study was to relate CA levels in patients with sickle cell disease compared to healthy controls in order to explore its use as a surrogate marker for the progression of clinical complications and treatment interventions.

MATERIALS AND METHODS

This case-controlled study was carried out in a tertiary hematology clinic. We included children with history of sickle cell disease between the ages of 6 months to 12 years with a confirmed diagnosis of sickle cell disease as determined by Hb electrophoresis. We also recruited controls: normal children with confirmed HbAA phenotype matched according to gender and age. We excluded patients who had: history of blood transfusion in the past 3 months, diagnosed with other hemoglobinopathies and enzymopathies. A total of 40 participants (28 males and 12 females) were enrolled for the study with age ranging from 2 months to 12 years. 20 SCD patients (14 men and 6 females) and 20 control individual, who visited the surgical clinic for elective hernia repair, were randomly selected to match the SCD patients with regard to demographic data such as gender and age.

Blood was obtained from children with SCD and healthy controls into Ethylene Diamine Tetra Acetic Acid (EDTA) anticoagulant bottles and labeled with the study number of participants. Full hemogram was done using symex XT-2000i (symex corporation Kobe Japan). As a primary screening procedure, sickling test was also carried out on the same day of collection according to the method [13]. The remaining blood was centrifuged for 15 minutes at a speed of 1000g using Thermo Scientific CL 10 centrifuge to separate red blood cells and plasma. Plasma was reserved in labeled containers with study numbers and stored at 20ºC for analysis of carbonic anhydrase while the remaining blood pellets were vortexed for 5 seconds and stored between 2° and 8° in all the samples represented as 95.5 ± 5.91 and also elevated HbF of 2.6 ± 6.42. The hemoglobin phenotypes of all patients and controls were confirmed using an automated capillary electrophoresis MINICAP system according to the method [13].

Carbonic anhydrase II levels were determined by a 96 well microplate ELISA kit purchased from ELAB science biotechnology precoated with CAII antibody according to [14]. All reagents, working standard and samples were prepared; 100μl of standard or sample were added to each well. The liquid in each well was removed without washing. 100μl of Biotin-antibody was added to each well and incubated for 1 hour at 37ºC after which each well was aspirated and washed five times. 90μl of TBM substrate was added to each well and incubated for 15-30 minutes at 37ºC. 50μl of sulfuric acid solution was added and the optical density of each well determined using a micro plate reader set at 450nm.

ETHICAL CONSIDERATION

The study was approved by the Ethical Committee of the institution review board (GCH/ERB/VOLXV/38). Written informed consent was obtained from the parents of all participants before enrollment.

STATISTICAL ANALYSIS

Data was processed and stored in Microsoft Access 2000. Minitab version 17 was used for all statistical analysis. Descriptive statistics were established and the values were expressed as mean ± standard deviation (SD). Data from patients and controls were compared using the 2 sample t-test. Simple linear regression analysis was used to find the relationship between CAII, gender and hematological parameters in children with clinical complications of SCD. P-values ≤ 0.05 were considered as indicating statistical significance.

RESULTS

Demographic information of the sampled population

In this study, a total of 40 children were recruited. The children’s ages, gender and the sickle cell status were established. The participants were placed in two groups, which were 20 children with SCD and 20 healthy controls. Table 1 shows that equal number of males (14 males) and females (6 females) from both the children with SCD and control group were sampled. In the sampling of the children with SCD, 16 children had clinical complications defined as stroke, anemia, infection, abdominal pain, priapism and pain crisis and 4 children had observable complications. The results showed that the mean age of male children with sickle cell disease was 7.28 years while for the female with SCD was 8.80 years. In the control group, the average age of the males was 4.94 years and that of the female was 5.17 years.

Hematological parameters

Table 3 shows that, levels of RBC, Hgb and HCT were significantly higher in healthy controls than children with SCD. The levels of WBC, Plts and MCV were significantly higher in children with SCD than in healthy controls. However, there was no significant difference in the levels of MCH and MCHC in both the groups. All the individuals with SCD screened were of the homozygous state Hb SS. This was characterized by raised HbS with a mean of 93.7 ± 5.91 and also elevated HbF of 2.6 ± 6.42. The HbA2 was moderately elevated (3.7 ± 3.5) as compared to the healthy controls. All healthy controls had normal hemoglobin HbAA with the percentage of HbaA in all the samples represented as 95.5 ± 2.9, while the average percentage of HbA2 was 3.19 ± 0.32 and HbF 1.31 ± 1.6 as shown in Table 2.

Carbonic anhydrase II levels

Sickle cell disease children had a significantly higher mean CAII levels than healthy controls (116.4 ± 50.03ng/ml vs 77.6 ± 42.29ng/ml, p=0.0001) (Table 3).
Table 1: Demographic information of the participants.

<table>
<thead>
<tr>
<th>Demography</th>
<th>SCD(n=20)</th>
<th>Controls(n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Percentage</td>
</tr>
<tr>
<td>Male</td>
<td>14</td>
<td>70</td>
</tr>
<tr>
<td>Female</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>Ages(Years)</td>
<td>Male Male</td>
<td>Female Female</td>
</tr>
<tr>
<td></td>
<td>7.28±1.08</td>
<td>8.80±1.88</td>
</tr>
<tr>
<td>Complications</td>
<td>13</td>
<td>3</td>
</tr>
</tbody>
</table>

SCD: Sickle Cell Disease; SD: Standard Deviation; n: Number of Patients

Table 2: Hemoglobin Electrophoresis.

<table>
<thead>
<tr>
<th>Variables</th>
<th>HbA%</th>
<th>HbA2%</th>
<th>HbF%</th>
<th>HbS%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children with SCD</td>
<td>Mean±SD</td>
<td>Mean±SD</td>
<td>Mean±SD</td>
<td>Mean±SD</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>95.5±2.9</td>
<td>3.19±0.32</td>
<td>2.6±6.42</td>
<td>93.7±5.91</td>
</tr>
</tbody>
</table>

Hb: Hemoglobin; HbA: Hemoglobin Adult; HbF: Hemoglobin Fetal; HbS: Hemoglobin Sickle; SD: Standard Deviation

Table 3: Mean values of hematological parameters.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Children with SCD</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Hgbg/dL</td>
<td>3.31±0.955</td>
<td>4.88±0.477</td>
</tr>
<tr>
<td>HCT %</td>
<td>9.07±0.157</td>
<td>12.75±1.187</td>
</tr>
<tr>
<td>MCV</td>
<td>27.29±5.11</td>
<td>37.66±3.341</td>
</tr>
<tr>
<td>MCH pg</td>
<td>85.25±15.65</td>
<td>77.28±3.996</td>
</tr>
<tr>
<td>MCHC g/dL</td>
<td>85.25±15.65</td>
<td>77.28±3.996</td>
</tr>
<tr>
<td>WBC</td>
<td>28.48±6.49</td>
<td>26.125±1.140</td>
</tr>
<tr>
<td>Plts</td>
<td>533.0±206.5</td>
<td>382.3±73.0</td>
</tr>
</tbody>
</table>

SD: Sickle Cell Disease; RBC: Red Blood Cell; Hgb: Hemoglobin; HCT: Hematocrits; MCV: Mean Cell Volume, MCH: Mean Cell Hemoglobin; MCHC: Mean Cell Hemoglobin Concentration; WBC: White Blood Cells; Plts: Platelets; *- significance at p≤0.05; t: 2 sample t test.

Correlation between erythrocyte carbonic anhydrase II and the hematological parameters

There was an inverse association with hemoglobin and MCV in children with SCD compared to the control group (Table 4).

Carbonic anhydrase II levels in relation to Gender

There was no significant difference in the mean levels of CAII recording according to gender in the cases (118.3 ± 51.1 vs 111.9 ± 48.5ng/ml p=0.652) while in the control group a significant difference in the mean levels of CAII with gender (87.8 vs 55.2ng/ml p=0.0001) was observed (Table 5).

Carbonic anhydrase in relation to clinical complications of sickle cell disease

Carbonic anhydrase II concentration was significantly enhanced in sicklers who suffered from clinical complications compared to sicklers who did not suffer from any clinical complications (126.2 ± 51.3 vs 79.8ng/ml, p=0.0001) (Table 6).

Relationship between carbonic anhydrase, hematological parameters and gender in children with clinical complications of sickle cell disease

Simple linear regression analysis showed a significant strong positive relationship between gender, CAII and the clinical complications of sickle cell disease (r=0.808, p=0.0001). Relationship between carbonic anhydrase II, hematological parameters and gender in children with clinical complications of sickle cell disease (r=0.808, r²=0.653, p=0.0001) is shown in Table 7 and 8.

DISCUSSION

This study investigated CAII levels among children with SCD. The mean CAII level was found to be significantly higher in sickle cell patients than the control group (116.4 ± 50.03 vs 77.6 ± 42.29, p=0.0001). A correlation of CA activity with erythrocyte zinc content has been suggested in other studies with an increase in both zinc and carbonic anhydrase in some patients with chronic leukemia and sickle cell anemia noted [12]. High levels of
CA activity in sickle cell patients have also observed by [10,12]. However, this did not provide evidence for the cellular expression or location of CA in the erythrocyte membrane.

Since peripheral mature non-nucleated erythrocytes apparently do not synthesize CA [15], increased CAII could have occurred only when young cells were produced to compensate for the decrease of CAI and/or decrease of band 3 protein concentration. Chiang [16], suggested that G6PDH-deficient patients suffered from hemolytic anemia hence had a high population of young erythrocytes which synthesized CAI at a slower rate compared to hemoglobin and further suggested that increased CAII compensated for decreased expression of CAI and decreased band 3 proteins. Increased CAII could have also resulted from surface expression (decryption) rather than an increase in CAII synthesis. Possibly CAII on the inner leaflet of the red blood cell or the cytoplasmic domain of band 3 was
translocated to the outer leaflet exposing the previously hidden enzyme [17,18].

According to this study red blood cell indices were generally low among the children with SCD compared to healthy controls with hemoglobin phenotype AA. Similar finding were obtained by [19], who found low red blood cell indices in the cases compared to the controls. High MCV levels were found in SCD patients in this study and were similar to the findings by Diop [20], Sadarangani and Serjeant [21]. These findings suggested macrocytic anemia due to high level of MCV and could be attributed to the effect of anemia of chronic disease, infection and hemolysis as supported by Akinbami [2].

Though not significant, a correlation between CAII and the hematological findings indicated an inverse association between CAII, hemoglobin and MCV. An early study indicated an inverse relation between CA and the fall in hemoglobin and suggested that hemoglobin and CA were structurally discrete though functionally related [22]. These findings could have suggested increased CAII levels in children with anemia resulting from ongoing hemolysis. WBC demonstrated a parallel association with CAII. Association between CAII and WBC could have also suggested increased CAII in chronic hemolysis. The study indicated no significant difference in the levels of CA II between gender in the SCD group. However, a significant difference in the levels of carbonic anhydrase II between gender was observed in the control group. Though experimental studies have proved that both androgen and estrogen influence the production of CA II [23]. Sickle cell disease is known to affect both male and female equally with gender differences experienced in adulthood [24]. The findings suggested that gender did not alter CAII levels in children with sickle cell disease.

This study indicated a significant high level of CAII in children who suffered from clinical complications of sickle cell disease compared to children who did not suffer from clinical complications of sickle cell disease. It is well known that the erythrocyte membrane undergo perturbation under hypoxia induced sickling exposing the previously hidden band 3 peptides which are recognized by surface antigens [18]. This leads to premature destruction of erythrocytes hence hemolysis a hallmark of the clinical complication. Mohamed & Ronquist [25], confirmed the reduction of band 3 protein in sickle cell patients and related it to the degree of hemolysis and so the degree of anemia. Increased CAII has been linked to decreased CAI and decreased band three proteins in hemolytic anemia [16].

Band three is considered to play a central role in erythrocyte membrane skeletal stability [25]. Waugh [26], suggested that denatured hemoglobin associated tightly with the cytoplasmic domain of the predominantly spinning protein, Band 3 and that the functional and ultra-structural abnormalities of sickle cell may have in some way been related to the membrane structural change. Since CAII is one of the associated structures of band 3 membrane components [27], an abnormality in the band 3 due to conformational change in the erythrocyte membrane, or perturbation could have resulted in description of CAII during the clinical complications of sickle cell disease. A possibility was demonstrated by the movement of phosphatidyl serine from the inner leaflet to the outer leaflet of the RBC membrane [18]. The findings of this study suggested that increased CAII during clinical complications could have been due to a young cell population resulting from hemolysis or decryption of CAII.

A strong significant positive relationship was obtained between gender, CAII and the clinical complication of sickle cell disease (r = 0.613, p= 0.018). Significantly high WBC, MCV with decreased HCT contributed to enhanced CAII levels in children with clinical complications of SCD. This was expected since sickled red blood cells have abnormal hemoglobin which is broken down at a faster rate than replacement (hemolysis). Low RBC has been associated with low hemoglobin and low hematocrit and could have been attributed to ongoing hemolysis [2]. Elevated WBC was expected in children with clinical complications since sickled RBC were unable to traverse the opening in the splenic cords and were phagocytosed and destroyed by macrophages [2]. The hematological findings suggested anemia due to ongoing hemolysis which contributed to enhance CAII due to the young population of RBC. Gender did not contribute significantly to high CAII in children with clinical complications of SCD and this could be attributed to the fact that sickle cell affected males and females equally [24].

CONCLUSION

Plasma CA II levels were significantly enhanced in children with sickle cell anemia. Gender did not influence CA II levels in SCD children. The mean carbonic anhydrase II levels were enhanced in children with clinical complications of sickle cell anemia compared with children without clinical complications.

LIMITATION OF THE STUDY

Due to financial constraints the study was carried out in one center with a small sample size therefore might not represent the entire population.

ACKNOWLEDGEMENTS

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REFERENCES
