A study to understand the relation between fetal haemoglobin, the hematological parameters and Xmn i gene polymorphism

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Abstract
Sickle cell disease is a public health issue in state of Chhattisgarh. The study focused on three aspects of sickle cells disease. Firstly erythrocyte levels of Hb F (measured by HPLC) was determined and correlated with various hematological indices (measured by cell counter). Secondly Hb F was correlated with Xmn I gene polymorphism of G γ gene using technique of PCR followed by gel electrophoresis. Thirdly mutant beta chain of Hb SS was distinguished from normal beta chain of Hb AA by Liquid chromatography Mass Spectrometry. We found no significant correlation between HbF and other hematological indices. Xmn I RFLP associated with G γ gene has correlation with Hb F levels in SS patients. Mutations in beta chain, particularly SS, are routinely detected by HPLC. Liquid Chromatography Mass Spectrometry can prove to be a viable option for detection of mutations in beta chain, particularly HbSS since mass difference between mutant chain and normal chain is wide enough to be sensitively detected by mass spectrometer.

Keywords: Fetal haemoglobin, Xmn 1, Sickle cell, Hematological Parameters, Hb SS

1. Introduction

Hemoglobin is the main substance of the red blood cell. It helps red blood cells carry oxygen from the air in our lungs to all parts of the body. Normal red blood cells contain hemoglobin A. Hemoglobin S and hemoglobin C are abnormal types of hemoglobin. Normal red blood cells are soft and round and can squeeze through tiny blood tubes (vessels). Normally, red blood cells live for about 120 days before new ones replace them. Sickle cell disease is an inherited blood disorder that affects red blood cells. People with sickle cell disease have red blood cells that contain mostly hemoglobin S, an abnormal type of hemoglobin. Sometimes these red blood cells become sickle-shaped (crescent shaped) and have difficulty passing through small blood vessels. When sickle-shaped cells block small blood vessels, less blood can reach that part of the body. Tissue that does not receive a normal blood flow eventually becomes damaged. This is what causes the complications of sickle cell disease. There is currently no universal cure for sickle cell disease (Richard et al., 1949).

People with sickle cell conditions make a different form of hemoglobin A called hemoglobin S (S stands for sickle). Red blood cells containing mostly hemoglobin do not live as long as normal red blood cells (about 16 days). They also become stiff, distorted in shape and have difficulty passing through the body’s small blood vessels. When sickle-shaped cells block small blood vessels, less blood can reach that part of the body. Tissue that does not receive a normal blood flow eventually becomes damaged.

The term sickle cell anemia was first used by Mason in 1922. He described the sickle cell shaped of red cells in three African American patients and proposed, based on case histories that the disease was hereditary in nature. Neel in 1949 used conventional family history analysis and speculated on the genetic basis of the trait. He claimed a homozygous inheritance of sickle cell disease and heterozygous inheritance of the trait. Four months later Linus Pauling and his co-workers presented that the hemoglobin molecule in sickle cell patient had a different electric charge compared to normal.

Electrophoresis demonstrated that the sickle cell hemoglobin had an altered molecular structure (Knight Julian, 2009).

It is most common in West and Central Africa where as many as 25% of the people have sickle cell trait and 1-2% of all babies are born with a form of the disease. In the United States with an estimated population of over 270 million, about 1,000 babies are born with sickle cell disease each year. Approximately 70,000 - 100,000 individuals in the United States have sickle cell disease and 3 million have sickle cell trait. In contrast, Nigeria, with an estimated population of 90 million in 1997, 45,000-90,000 babies with sickle cell disease are born each year. The transatlantic slave trade was largely responsible for introducing the sickle cell gene into the Americans and the Caribbean. However, sickle cell disease had already spread from Africa to Southern Europe by the time of the slave trade, Hence is present in Portuguese, Spaniards, French Corsicans, Sardinians, Sicilians, mainland Italians, Greeks, Turks and Cypriots. Sickle cell disease appears in most of the Near and Middle East countries including Lebanon, Israel, Saudi Arabia, Kuwait and Yemen. The condition has also been reported in India and Sri Lanka. Sickle cell disease is an international health problem and truly a global challenge (Richard et al., 1949).

1.1 Causes

Sickle cell anemia is an inherited disease. People who have the disease inherit two genes for sickle hemoglobin—one from each parent. Sickle hemoglobin causes red blood cells to develop a sickle, or crescent, shape. Sickle cells are stiff and sticky.
They tend to block blood flow in the blood vessels of the limbs and organs. Blocked blood flow can cause pain, serious infections, and organ damage (Knight Julian, 2009).

### 1.2 Molecular basis of sickle cell disease

Sickle cell disease is an inherited structural disorder of hemoglobin. The disorder specifically involves the β globin subunit of the hemoglobin molecule such that instead of the normal adult hemoglobin \( Hb \alpha(2)\beta(2) \), \( Hb \delta(2)\gamma(2) \), a single amino acid substitution from glutamic acid to valine results in \( Hb \delta(2)\gamma(2) \). Glutamic acid is negatively charged while valine is hydrophobic; the amino acid change in each β globin chains in the hemoglobin molecule promotes hydrophobic contact with alanine, phenylalanine and leucine residues in adjacent molecules such that there is reversible association in conditions of deoxygenation forming 14 stranded polymers which can cross link, the long fiber stretching and deforming the red blood cells (http://www.academon.com/Research-Paper-Sickle-Cell-Anemia/94492).

People who inherit a sickle hemoglobin gene from one parent and a normal gene from the other parent have a condition called sickle cell trait. Their bodies make sickle Hemoglobin and normal hemoglobin. People who have sickle cell trait usually have few, if any, symptoms and lead normal lives. However, some people may have medical complications. People who have sickle cell trait can pass the sickle hemoglobin gene to their children (http://www.sickle-thal.nwlh.nhs.uk/Blood/HaemoglobinF.aspx).

In order for sickle cell anemia to manifest itself the presence of two defective genes (SS) are needed. In other words, if two parents are the carriers of one sickle hemoglobin gene (s) as well as a single normal cell (A) then each child born from these parents will have a 25% chance of inheriting two defective genes and having sickle cell anemia; a 25% chance of inheriting two normal genes and not having the disease; and a 50% chance of being an unaffected carrier like the parents. Individuals who have only one copy of the mutation are said to have sickle cell trait. These people are usually healthy but can transmit the disease to their children. This aspect is clarified by the fact that, Sickle Cell trait (AS) is an inherited condition in which both hemoglobin A and S are produced in the red blood cells, always more A than S. Sickle cell trait is not a type of sickle cell disease. People with sickle cell trait are generally healthy (http://www.nhlbi.nih.gov/health/dci/Diseases/Sca/SCA_Causes.html).

### 1.3 Fetal haemoglobin and SCD

During life in the womb and from very early in pregnancy every unborn baby produces Fetal Hemoglobin (Hb F), this is irrespective of the type of Adult Hemoglobin they have inherited from their parents. At birth the level of Fetal Hemoglobin is high whilst the level of Adult Hemoglobin is low, but by one year of age there is a switch over between the level of Adult and Fetal hemoglobin. (Donalson et al., 2001)

For example, a person who has inherited normal adult Hemoglobin AA from their parents will have the following approximate Fetal, Adult and A2, hemoglobin levels:

- A person who has inherited Sickle Cell Trait (Hb AS) from their parents will have the following approximate Fetal, Adult and A2 haemoglobin levels:
  - Fetal Haemoglobin is normal hemoglobin and is very efficient at carrying oxygen, even more efficient than adult haemoglobin, which is why it is very useful during fetal life when the unborn baby is totally dependent on its mother for oxygen. Without Fetal Haemoglobin the unborn baby cannot develop or survive in the womb and such a fetus will die in the early stages of pregnancy.
  - High fetal hemoglobin (HBF) levels are believed to ameliorate the manifestations of homozygous sickle cell (SS) disease. The corollary implies that patients with low HBF levels should have more severe clinical courses (Maria Proytcheva, 2011). In sickle cell disease (SCD), an increase in HBF inhibits the polymerization of sickle hemoglobin and the resulting pathophysiology (Menzel Stephan & Their Swee Lay, 2009).
  - Neonates with sickle cell anemia appear well at birth because the majority of the total haemoglobin consists of haemoglobin F which produces normal RBCs, the hematologic manifestations of the disease becomes apparent at 19 - 12 weeks of age when the transcription of the abnormal beta chain becomes significant (Swee lay Thi et al., 2009).

In humans, a shift from γ- to β-globin gene expression around birth, underlies the switch from fetal to adult hemoglobin (Hb) Production. Increased levels of fetal hemoglobin (Hbf, α_{γ}) are of no consequence in healthy adults, but confer major clinical benefits in patients with sickle cell anemia (SCA) and β thalassemia, diseases that represent major public health problems. Genetic studies have shown that individuals with hemoglobinopathies concurrent with high expression of HBF can maintain normal fitness levels that would otherwise be severely limited by the debilitating consequences of their disease. Inter-individual HBF variation is largely genetically controlled, with one extreme caused by mutations involving the β globin gene (HBB) complex, historically referred to as pancellular hereditary persistence of fetal hemoglobin (HPFH). Xmn-1 polymorphism is a factor, which increases fetal hemoglobin production (Durack-Bown et al., 1999).

These Mendelian forms of HPFH are rare and do not explain the common form of heterocellular HPFH which represents the upper tail of normal Hbf variation, and is clearly inherited as a quantitative genetic trait. Genetic studies have identified three major quantitative trait loci (QTLs) (Xmn1- HBG2, HBS1L-MYB intergenic region on chromosome 6q23, and BCL11A on chromosome 2p16) that account for 20 - 50% of the common variation in Hbf levels in patients with SCA and β thalassemia, and in healthy adults. N 1985, a polymorphism (C/T at position-158 of HBG2, later termed Xmn1 - HBG2

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or rs7482144) was identified from re-sequencing of the HBG genes, and shown to promote the expression of HBG2, and to contribute to HbF variability (Bailey et al., 1992).

Epidemiologic studies have shown FCs to be influenced by age, sex, and a DNA sequence variant (CT) at position-158 upstream of the G-globin gene, referred to as the XmnI-G polymorphism. The XmnI-G polymorphism is common in several populations; the “T” variant, which creates a cleavage site for the XmnI restriction enzyme, has been shown to be associated with increased FC level (Kar et al., 1986). Bailey et al. (1992) in their paper Fetal haemoglobin and early manifestations of homozygous sickle cell disease found the relevance of fetal haemoglobin (HbF) concentration to the development of early clinical manifestations of homozygous sickle (SS) disease, they investigated by examining the time of first occurrence and the proportional hazard of these complications in three groups of the HbF distribution at age 5 years. The relationship suggested that a critically low HbF concentration increased the risk, little difference in risk occurring between the medium and high HbF groups. The abdominal painful crisis and hypersplenism were not related to HbF concentration suggesting that the degree of sickling may not be important in their genesis (Donaldson et al., 2001).

2. Materials and method

Subjects were 30 sickles homozygous sickle cell anemia patients. About 5 ml blood sample was collected from these patients after taking their consent. Study was approved from institutional ethical committee. Clinical evaluation was done during physical examination (visually appeared) as well as laboratory evaluation. Complete blood count and red cell indices were measured. Quantitative assessment of hemoglobin Hb F, Hb A, Hb A2 and Hb S and diagnosis of HbSS was performed by ion exchange chromatography and mass Spectrometry (LC-MS). DNA extraction done by phenol chloroform method and DNA quantification done by UV Viz spectrophotometer. Xmn1 polymorphism analysis was done by PCR-RFLP method as per Sutton et.al (1989). ANOVA test was applied to analysis of variance between groups.

3. Results and discussion

As is evident from table, there is no significant difference in hematological parameters between any of the groups. Fetal hemoglobin is one of the major factors that alter the clinical course of disease. Studies have shown that high HbF levels are associated with milder disease (Merghouh et al., 1997) whereas Many workers has noted benign disease where HbF levels are high (http://www.sicklecelldisease.org/index.cfm page about-scd). Fetal hemoglobin (HbF) genes are genetically regulated and the level of HbF and its distribution among sickle erythrocytes is highly variable.

Table. 1. Approximate value of Fetal, Adult and A2, hemoglobin levels of a person who has inherited normal adult Hemoglobin AA from their parents

<table>
<thead>
<tr>
<th>Haemoglobin</th>
<th>Newborn</th>
<th>&gt; 1 Year Old to Adulthood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin F</td>
<td>90%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Haemoglobin A</td>
<td>5 – 10%</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>Haemoglobin A2</td>
<td>2.2 – 3.5%</td>
<td>2.2 – 3.5%</td>
</tr>
</tbody>
</table>

Table. 2. Correlation of HbF with various hematological parameters

<table>
<thead>
<tr>
<th>Group</th>
<th>Hb F %</th>
<th>N</th>
<th>AGE</th>
<th>Hb</th>
<th>WBC</th>
<th>MCH</th>
<th>MCV</th>
<th>MCHC</th>
<th>HCT</th>
<th>MCH</th>
<th>RBC count</th>
<th>RDW</th>
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<tbody>
<tr>
<td>&lt;10</td>
<td>Mean</td>
<td>11</td>
<td>9.09</td>
<td>5.5</td>
<td>14.8</td>
<td>78.2</td>
<td>34.4</td>
<td>20.9</td>
<td>26.7</td>
<td>3.24</td>
<td>23.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SD±</td>
<td></td>
<td>2.7</td>
<td>10.3</td>
<td>1.8</td>
<td>7.5</td>
<td>4.2</td>
<td>7.5</td>
<td>2.6</td>
<td>15.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-20</td>
<td>Mean</td>
<td>19</td>
<td>8.1</td>
<td>7.4</td>
<td>17.3</td>
<td>80.9</td>
<td>33.2</td>
<td>22.2</td>
<td>27.0</td>
<td>2.8</td>
<td>22.7</td>
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<tr>
<td></td>
<td>SD±</td>
<td></td>
<td>1.9</td>
<td>9.9</td>
<td>3.6</td>
<td>3.6</td>
<td>4.0</td>
<td>3.6</td>
<td>0.9</td>
<td>14.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;20</td>
<td>Mean</td>
<td>15</td>
<td>7</td>
<td>6.3</td>
<td>13.8</td>
<td>80.62</td>
<td>32.8</td>
<td>20.6</td>
<td>26.2</td>
<td>2.5</td>
<td>19.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SD±</td>
<td></td>
<td>2.3</td>
<td>7.2</td>
<td>6.7</td>
<td>4.1</td>
<td>8.1</td>
<td>3.2</td>
<td>0.9</td>
<td>12.6</td>
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</table>

HbF is the major genetic modulator of the hematological and clinical features of sickle cell. The effect of −158 C > T mutation on expression of Gγ globin gene has been the subject of considerable interest. In the present study fetal hemoglobin was measured by cation exchange chromatography while other hematological indices as hemoglobin concentration, red blood cells count, white blood cells count, MCV, MCHC, HCT, MCH, RDW were measured using cell counter.
Patients were divided into three groups as shown in table number 6 based on HbF concentration in whole blood. Group I, II, III had < 10, 10-20 & > 20 percent HbF respectively. The present study does not show any alterations in hematological parameters in different groups. But, Donaldson et al in 2001 have reported that there was a positive trend between HbF grouping and Hb, PCV, MCH.

Solubility test has been a reliable screening test for sickle cell disease while cellulose acetate electrophoresis has been used as a confirmatory test for distinguishing homozygous and heterozygous sickle cell disease with normal individuals. In our study we also find these two tests to be extremely reliable, fast and technically not demanding.

In the present study we set out and managed to correlate HbF levels in SS patients with Xmn I polymorphism in Gγ gene. Earlier studies have observed relationship between Xmn I polymorphism and Fetal hemoglobin expression as measured by F-cell levels in sickle cell trait (http://www.nhlbi.nih.gov/health/dci/Diseases/Sca/SCA_Causes.html). The Xmn I polymorphism at -158 position of the Gγ gene was confirmed by Xmn I restriction enzyme digestion of a 650 bp amplified DNA sequence from the promoter region of the Gγ gene.

![Fig.1. PCR](image)

Lane 1 - 100 bps Ladder  
Lane 2- Blank  
Lane 3- Undigested 650 bps sequence  
Lane 5-8 - DNA samples from patients showing digested bands of 650bp

4. Conclusion

HbF is not significantly correlated with hematological parameters as hemoglobin concentration, white blood cells count, mean corpuscular volume, mean corpuscular hemoglobin concentration, hematocrit, mean corpuscular hemoglobin, RBC count, red cell width. Xmn I RFLP associated with Gγ gene has correlation with Hb F levels in SS patients. Mutations in beta chain, particularly SS, are routinely detected by HPLC. Liquid Chromatography -Mass Spectrometry can prove to be a viable option for detection of mutations in beta chain, particularly HbSS since mass difference between mutant chain and normal chain is wide enough to be sensitively detected by mass spectrometer.

5. References

1• An in-depth look at sickle cell anemia. Written in 2006; 2,929 words; http://www.academon.com/Research-Paper-Sickle-Cell-Anemia/94492
5• http://asheducationbook.hematologylibrary.org/cgi/content/full/2006/1/58
7• http://www.sicklecelldisease.org/index.cfm page about-scd


15. Swee lay Thien, Stephan Menzel, Mark Lathop and Chad Garner (2009) Control Of Fetal Hemoglobin: New Insights Emerging From Genomics And Clinical Implication. By, Division Of Gene And Cell Based Therapy, King's College London School of Medicine, Molecular Haematology, Denmark Hill Campus, London SE5 9NU, UK,