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REVIEW

Clinical biomarkers in sickle cell disease

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Abstract Sickle cell disease (SCD) is a hereditary blood disorder caused by a single gene. Various blood and urine biomarkers have been identified in SCD which are associated with laboratory and medical history. Biomarkers have been proven helpful in identifying different interconnected disease-causing mechanisms of SCD, including hypercoagulability, hemolysis, inflammation, oxidative stress, vasculopathy, reperfusion injury and reduced vasodilatory responses in endothelium, to name just a few. However, there exists a need to establish a panel of validated blood and urine biomarkers in SCD. This paper reviews the current contribution of biochemical markers associated with clinical manifestation and identification of sub-phenotypes in SCD.

Contents

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1. Introduction

Sickle cell disease (SCD) or sickle cell anemia (SCA) is an inherited disorder of hemoglobin (Hb) caused by substitution of a single nucleotide from thymine to adenine (GAG → GTG) in the β-chain of hemoglobin resulting in amino acid valine instead of glutamic acid (Rees et al., 2010). This point mutation is responsible for alteration in the properties of the hemoglobin tetramer, with a tendency to polymerize in the deoxygenated state (Ballas, 2002), altering normal, flexible biconcave shaped red blood cells (RBCs) into stiff, rigid, sickle cell. The rate of polymerization of Sickle cell hemoglobin (HbS) is directly related to fundamental pathophysiology of hemolytic anemia and vaso-occlusion (Samuel et al., 1990).

1.1. Biomarkers in SCD

The Biomarkers Definitions Working Group has defined a "biomarker" as a "characteristic that is objectively measured and evaluated as an indicator of normal biological or pathogenic processes, or pharmacological responses to a therapeutic intervention" (Biomarkers Definitions Working Group, 2001). There is high variability in terms of severity of SCD. Different major complications of SCD such as acute chest syndrome (Vichinsky et al., 2000), cerebrovascular disease (Ohene-Frempong et al., 1998), kidney failure (Scheinman, 2009) and early deaths (Platt et al., 1994) lead to variability in biomarkers.

Meaningful markers therefore have prime importance in the management of this disease. In this review, we will focus on the analysis of body fluids and tissues that have prime importance in the management of SCD. DNA markers (Steinberg and Adewoye, 2006) and imaging biomarkers will not be discussed in this review although they have been shown to be beneficial in the management of SCD (Adams et al., 1998).

1.2. Biomarkers used for HbS polymerization

Polymerization of the deoxygenated HbS is the leading phenomenon contributing to SCD related medical problems. The polymerization initiation rate is directly proportional to quantity of deoxygenated HbS within the RBC. Currently such biomarkers which can directly measure HbS polymerization

Table 1 Effect of α-thalassemia on some clinical complications of sickle cell anemia (adapted from Steinberg, 2009).

Sickle cell disease phenotype	Effect of α-thalassemia			
	Protective[a]	Permissive[b]	Unrelated	Probably little effect
Survival	+	–	–	–
Painful episodes	–	+	–	–
Stroke	+	–	–	–
Osteonecrosis	–	+	–	–
Acute chest syndrome	–	–	+	–
Cholelithiasis	+	–	–	–
Leg ulcer	+	–	–	–
Splenic function	+	–	–	–
Growth and development	–	–	+	–
Menarche	+		–	–
Priapism	–	+	–	–
Splenic sequestration	–	–	–	+
HbF	–	–	–	+

[a] "Protective" denotes a reduction in the incidence or prevalence of a phenotype with α-thalassemia.
[b] "Permissive" denotes an increased incidence or prevalence of a phenotype when α-thalassemia is present.

rate in RBCs do not exist. It is not clear whether any direct relation exists between SCD phenotypic diversity and different rates of HbS polymerization (Vekilov, 2007). Fetal hemoglobin (HbF) and α-thalassemia are the two well-studied biomarkers in SCD, capable of changing the intracellular concentrations of HbS that ultimately dictate the pace and extent of polymerization.

1.2.1. HbF

HbF is different from the adult hemoglobin due to the higher affinity of HbF to bind oxygen, hence providing the growing fetus with better access to oxygen from the mother's bloodstream. It has been shown clinically that cells containing HbF survive longer than cells with HbS (Charache, 1990). Polymerization of HbS is the pivotal step in the pathophysiology of SCD but elevated levels of HbF have been shown to halt this process (Franco et al., 2006; Lanzkron et al., 2008) . Furthermore, due to its inability to enter the HbS-polymerization phase, HbF can be regarded as the pivotal regulator of the clinical and hematologic features of SCD (Rees and Gibson, 2012). Moreover, Sebastiani et al. have defined the "HbSF" phenotype as the HbF concentration of 10% or more in SCD patients who are aged four or above because this is the age at which levels of HbF become stable (Sebastiani et al., 2010).

Level of expression of HbF varies in different variants of sickle erythrocytes, of which Saudi-Indian and Senegal haplotype of HBB-like gene cluster (Homozygous for Glu6Val in β-globin gene) have the highest levels. Levels of HbF differ in other variants of SCD. In terms of the prevalence of SCD in Saudi Arabia, mostly individuals from the Eastern Province are the carriers of the Saudi-Indian β-globin gene-like cluster haplotype with elevated levels of HbF, and a milder clinical manifestation of SCD symptoms. Southwestern Province patients, on the other hand, are the carriers of the typical *HBB* African-derived haplotypes with lower levels of HbF. In addition, they exhibit lower incidences of ischemic brain injury, priapism, leg ulcers and increased incidences of splenomegaly in comparison with African Americans phenotype. This difference has been attributed to variation in levels of HbF or joint occurrence of α-thalassemia (Akinsheye et al., 2011).

1.2.2. Co-inheritance of α-thalassemia

α-Thalassemia is a form of genetic blood disorder in which there is impaired production of alpha globin chains of hemoglobin, eventually resulting in anemia. One in three persons with SCD from the American black population carries α-thalassemia variant with different complications. In people of African descent, there often exists heterozygosity or homozygosity for the deletion of the α-globin gene. α-Thalassemia regulates sickle cell anemia by reducing the rate of polymerization, thereby lowering intracellular concentrations of HbS. The hematologic and laboratory variations observed clinically in SCA-α-thalassemia patients include increased hemoglobin concentration, lower mean corpuscular volume (MCV), lower reticulocyte count, elevated HbA2, lower bilirubin levels, fewer aggregated and irreversibly-sickled cells, increased erythrocyte lifespan, and almost no change in HbF concentration. The extent of these changes is linked to the number of deleted α-globin genes (Steinberg, 2009). The benefits and liabilities associated with the presence or absence of α-thalassemia in SCA are most probably due to the effects of α-thalassemia on cell hemolysis. Some associations of α-thalassemia and the clinical features of sickle cell anemia are summarized in Table 1:

1.3. Biomarkers used for red blood cell dehydration

RBCs in SCD are typified by bizarrely increased cation permeability and cations loss which causes RBC dehydration, and increases the rate of Hb polymerization. To assess the cation loss, fresh blood and radioactive isotopes are needed, hence making it difficult to use as routine biomarker. However, indirect markers of RBC dehydration are measurement of irreversibly sickled cells (ISCs) on the blood film as well as measurement of red cell density (Ellory et al., 1998).

ISCs exist in the form of elongated, sharpened-end cells with cell axial ratio (length divided by width) close to 2. Even when fully oxygenated, the ISCs tend to maintain their atypical shape. Studies have found no significant relationship among the numbers of ISCs with acute complications (Rodgers et al., 1985). Those RBCs were observed as a wet preparation when imaged with phase contrast microscopy.

Likewise, high density gradient centrifugation or cytometry is used as a biomarker for red cell density. Some studies have suggested that the increase in pain is associated with higher number of dense red cells (Ballas and Smith, 1992).

1.4. Biomarkers for red cell adhesion

Structural asymmetry of RBC membrane, which is maintained normally, is lost in SCD, causing the exposure of erythrocyte phosphatidylserine and phosphatidylethanolamine as revealed by flow cytometry analysis. In typical SCD, vascular occlusion is initiated by adhesion of circular reticulocytes to vascular endothelium. This leads to a cascade of painful crisis ending in organ failure. At least two receptors, usually present on reticulocytes, appear to be important in this adhesion process. The CD36 (Glycoprotein IV) is implicated in the binding of RBCs to endothelial lining of blood vessels by its binding to thrombospondin (Kaul et al., 2009). Likewise, the binding of integrin α4β1 (very late activation antigen-4) to vascular cell adhesion molecule-1 (VCAM-1) and fibronectin occurs on the endothelial lining of blood vessels. Flow cytometry studies have revealed that the expression of CD36 and integrin α4β1 is reduced on the reticulocytes of SCD patients when hydroxycarbamide is used, which may be a way to monitor therapeutic benefits. However, hereditary nonexistence of CD36 receptor has not been shown to change the manifestation of SCD, thus decreasing the value of red cell adhesion as a biomarker (Lee et al., 2001).

1.5. Biomarkers for white cell adhesion

Some studies have demonstrated that the incidences of acute pain in SCD patients increase when there is a heightened expression of certain leukocyte adhesion molecules such as L-selectin and αMβ2 (Okpala et al., 2002). This suggests that some white cell adhesion molecules can serve as biomarkers.

1.6. Biomarkers for inflammation

Increasing amount of experimental evidence suggests a close connection between chronic inflammatory processes and sickle cell disease (SCD) (Platt, 2000). Many markers of inflammation are increased in SCD and they may have clinical value as biomarkers. These markers of inflammation include.

1.6.1. Total white cell count

SCD is characterized by abnormally elevated total white cell count, secondary to hyposplenism and inflammation. Unusually high white cell count in SCD may be prognostic of conditions of clinical severity such as brain infarcts, acute chest syndrome and death. However, those SCD patients taking hydroxycarbamide show evidence of decreased white cell count and reduced frequency of acute pain (Charache, 1997).

1.6.2. C-reactive protein (CRP)

C-reactive protein (CRP) is found in the plasma. It is the most commonly assessed marker of acute and chronic inflammation. Acute chest syndrome (ACS) manifests in SCD patients when there is an increase in CRP levels due to vaso-occlusion, hence blocking the blood flow to organs and causing vaso-occlusion crisis (Bargoma et al., 2005). Krishnan et al. have also reported a strong link between high sensitivity C-reactive protein (hs-CRP) and vaso-occlusion in pediatric SCD patients (Krishnan et al., 2010).

1.6.3. Erythrocyte sedimentation rate (ESR)

The erythrocyte sedimentation rate (ESR) is the rate at which RBCs sediment or settle in a period of 1 h. ESR is a non-specific measure of inflammation. In SCD patients, the interpretation of ESR is complicated due to their low hematocrit values, which tends to give higher ESR values. This is further complicated by the fact that sickle erythrocytes fail to form rouleaux, which results in lower ESR values. This problem can be partially circumvented by increasing the reproducibility of this assay by repeated measurements in individual patients in order to observe inflammation processes. However, established normal ranges may still not apply in the case of SCD patients (Eastman, 1984).

1.6.4. Secretory phospholipase A2 (sPLA2)

The enzyme secretory phospholipase A2 (sPLA2) cleaves phospholipids and generates fatty acids (such as arachidonic acid) leading to a cycle of inflammatory events. It has been reported in a clinical study that elevated levels of sPLA2 are detected in ACS patients 24–48 h before the clinical diagnosis of ACS. It was shown that increased levels of sPLA2 can predict impending ACS in patients with sensitivity reaching up to 100% and specificity as high as 67% (Styles et al., 2000). Likewise, it has been shown in a small sample randomized, clinical trial blood transfusion lowered sPLA2 levels and prevented ACS in SCD patients, suggesting that sPLA2 can serve as a useful biomarker (Field et al., 2009; Styles et al., 2007).

1.6.5. Other inflammatory markers

The inflammatory nature of SCD is further confirmed in studies which measured other biomarkers such as elevated levels of interleukins (IL2, IL3, IL6, IL8 and IL10), urinary cysteinyl leucotriene E4 (28), and serum levels of prostaglandin-E2, CA 15-3, soluble CD40 ligand, HSP-70, ferritin, angiopoietin 1 and 2, stromal derived factor 1, tumor necrosis factor-α and tumor necrosis factor receptor-1 (Eastman, 1984).

2. Biomarkers for hemolysis

2.1. Hemoglobin concentration

Low hemoglobin is the solitary most useful biomarker in SCD. It is generally associated with unpredictable future clinical outcomes of SCD (Kato et al., 2006) with higher risk for medical problems most important of which is stroke either due to cerebral hemorrhage or cerebral infarction (Gueguen et al., 2014), premature death (Andemariam et al., 2014), abnormal transcranial Doppler (TCD) velocities (Rees et al., 2008), and high tricuspid regurgitant jet (TRJ) velocity (Gladwin et al., 2004).

2.2. Direct measurement of RBC survival

The typical RBC survival in SCD is 15–17 days with high variability in this range (Hebbel, 2011). Direct measurement of RBC survival by chromium-53 labeling is not feasible for regular clinical use. Nevertheless, biotin labeled RBC is more

useful after proper standardization of the assay (Mock et al., 2011).

2.3. Reticulocyte count

Reticulocyte count correlates well with RBC life span and is a routine procedure in automated blood cell counters. However, it is not clear whether it gives any additional information over total Hb values (Hebbel, 2011).

2.4. Serum lactate

Serum lactate dehydrogenase (LDH-1) levels increase in SCD patients with leg ulcers, priapism, and TRJ and TCD velocities. Although it is the total LDH that is measured in clinical practice, it has been observed that other LDH isoenzymes found in the brain, liver and muscle are reduced in SCD (Kato et al. 2006; O'Driscoll et al., 2008). LDH levels also tend to increase in erythropoiesis with premature death of RBCs and other cellular events which involve increased incidences of apoptosis. However, it remains ambiguous as to whether elevated levels of LDH are indicative of SCD pathology or it is just the outcome of increased anemia. Aspartate transaminase has similar prognosis as a biomarker in SCD, although not routinely used (Rees et al., 2008).

2.5. Haptoglobin

Almost all individuals with SCA have low levels of haptoglobin and this may not have any medical relevance (Moller et al., 2011). Under normal physiological conditions, free plasma hemoglobin can easily bind to haptoglobin and the levels of haptoglobin are undetectable under such conditions. On the other hand, due to the presence of intravascular hemolysis, the haptoglobin molecules are saturated and plasma hemoglobin levels are elevated in SCD. Measurements are technically challenging due to the sensitivity limits of the test involving extremely fresh blood sample. This is further complicated by the fact that it is exactly not apparent if what is being measured includes haptoglobin–hemoglobin complexes, haemopexin–haem complexes, or methemalbumin/hemoglobin in those microparticles (Kato et al., 2006). Although free plasma hemoglobin levels correlate with LDH (Hebbel, 2011), the use of haptoglobin as a clinical marker is still not documented.

3. Biomarkers of hypercoagulability

Hypercoagulability refers to the tendency to form blood clot inside a blood vessel and obstructing the blood flow due to certain inherited or acquired disorders. Hypercoagulability in SCD is complex with many factors involved in it. SCD patients exhibit elevated plasma levels of thrombin generation markers, abnormal activation of the fibrinolytic system, depletion of natural anticoagulant proteins and increased tissue factor expression, even in the non-crisis, stable condition of SCD (Ataga and Key, 2007). The process of coagulation is activated when there is enhanced exposure of membrane erythrocyte phosphatidylserine and erythrocyte phosphatidylethanolamine as well as enhanced circulating microparticles originating from numbers of erythrocytes, endothelial cells, white blood cells and platelets (Shet et al., 2003). Tissue factor expression on endothelial lining of blood vessels is induced by free haem. In addition, platelet activation is also enhanced during this process (De Franceschi et al., 2011). To prove that monitoring hypercoagulability is beneficial for clinical purposes needs further studies. CRP levels are always abnormally high in individuals with SCD and this information alone is not sufficient in the diagnosis of venous thromboembolism. Thrombosis has not been studied extensively in the clinical settings and the few studies that have been conducted on thrombosis claim that SCD represent a lesser degree of thrombophilia in which the prevalence of venous thromboembolism may not be enhanced (Stein et al., 2006). Currently there are no compelling reports demonstrating an association between the occurrences of vaso-occlusive complications and blood coagulation markers. Likewise, no studies to date have shown any clinical advantages of anticoagulant or antiplatelet therapies, even though there is a need to conduct randomized, clinical trials to demonstrate any benefits of such therapies (Eastman, 1984).

4. Biomarkers for oxidative stress

Oxidative stress appears to contribute to the pathophysiology of SCD. SCD has been linked with biological processes such as inflammation, vasculopathy, hemolysis, infection, vaso-occlusion, and reperfusion injury (Nur et al., 2011b). Therefore, SCD patients at high risk of oxidative damage can be identified by the use of biomarkers for oxidative stress. This can also help in the management of treatments involving the use of zinc, N-acetylcysteine, and vitamin supplements especially C and E (Jones, 2002).

Reduced glutathione (GSH) is a major tissue antioxidant. In the event of cells exposed to increased oxidative stress, GSH changes to oxidized glutathione (GSSH) and, hence, the ratio of GSH/GSSG decreases. In SCD patients, GSH levels as well as GSH/GSSG ratio are decreased (Stein et al., 2006; Moller et al., 2011). Therefore, measurement of GSH levels may serve as a useful biomarker of *in vivo* oxidative stress (Jones, 2002; Ashfaq et al., 2006). In addition, GSH and glutamine concentrations are among the indirect markers to assess the oxidative assault on tissues and cells in disorders such as SCD (Morris et al., 2008).

5. Biomarkers for vasculopathy and endothelial dysfunction

Stroke, pulmonary hypertension and renal disease are recognized as the most important complications of vasculopathy and endothelial dysfunction. The direct markers of vasculopathy depend on evaluation of the type and role of the vascular system by employing the ultrasound-based technique using flow-mediated endothelium-dependent dilatation (de Montalembert et al., 2007). Identified biomarkers for endothelial dysfunction which are abnormal in SCD patients are given as follows.

5.1. Triglycerides

Pediatric patients with SCD exhibit high triglyceride levels, which correlate considerably well with markers of hemolysis, such as LDH and FMD (Zorca et al., 2010).

5.2. Apolipoprotein A-1

SCD patients show diminished levels of apolipoprotein A-1 as identified by proteomic analysis when compared with same gender, healthy controls (0.98 g/L vs. 1.33 g/L, $P < 0.001$) (Yuditskaya et al., 2009).

5.3. Vascular endothelial growth factor (VEGF)

SCD is also characterized by elevated levels of VEGF, a marker indicating blood vessel damage and hypoxemia. Moreover, increased levels of VEGF correlate well with many other biomarkers signifying other problems such as hemolysis, inflammation and enhanced TRJ velocity (Ashfaq et al., 2006).

5.4. Placental growth factor (PGF)

PGF is produced by placental and erythropoietic tissue, particularly during embryogenesis. PGF plays a pivotal role in angiogenesis and activation of monocytes. SCD is characterized by elevated blood concentrations of PGF during steady-state with subsequent increase during the episodes of acute pain and/or during complications arising from pregnancy in SCD women. PGF can serve as an important biomarker linked with pregnancy in SCD (Brittain and Parise, 2007).

5.5. Endothelin-1

Endothelin-1 (ET-1) is a protein that is found in endothelial cells and also in other cell types. The presence of ET-1 and its receptors in neurons is an indication of its potential role as a neurotransmitter and neuromodulator. Studies utilizing exogenous ET-1 have demonstrated that pain transmission is influenced by ET-1 (Eastman, 1984). In SCD patients, the levels of ET-1 rise with simultaneous increase in the intensity of pain. However with hydroxycarbamide treatment, ET-1 levels decrease with decrease in pain (Lapoumeroulie et al., 2005).

6. Biomarkers of damage to specific organs

6.1. Bone disease

The most relevant clinical biomarkers to bone disease are Vitamin D and alkaline phosphatase. Low levels of Vitamin D are characteristically seen in pediatric SCD patients whereas increased alkaline phosphatase levels are observed with growth (Chapelon et al., 2009). Acute bone disease in SCD happens with vaso-occlusion and osteomyelitis whereas chronic bone disease occurs in the event of avascular necrosis of the hips and shoulders. The two biomarkers of bone turnover pyridinoline and deoxypyridinoline are composed of cross-linked collagen fibers. Urinary levels of both pyridinoline and deoxypyridinoline are elevated in asymptomatic SCD and these levels rise even further in the events of acute pain (Nebor et al., 2010).

6.2. Cardiac disease

Cardiac disease is considered unusual in SCD. However, cardiovascular problems in SCD patients have been shown to contribute to more than 15% of adult deaths (Gladwin et al., 2004).

6.3. Tricuspid regurgitant jet velocity (TRV)

Increased tricuspid regurgitant jet velocity (TRV) is linked to higher incidences of unexplained deaths in young adult SCD patients with cardiopulmonary complications (Machado et al., 2006).

6.4. NT-pro brain natriuretic peptide (NT-proBNP)

N-terminal prohormone of brain natriuretic peptide (NT-proBNP) is a protein generated by the heart and it is found in higher levels in patients suffering from congestive heart failure and pulmonary hypertension. NT-proBNP can also serve as a biomarker for SCD patients because its levels are elevated in those patients (Machado et al., 2006).

6.5. Cardiac troponin I

Cardiac troponin I levels are elevated in SCD individuals going through ACS, which is also accompanied with high TRV and increased death rates (Mekontso-Dessap et al., 2008).

6.6. Hyposplenism

Spleen is one of the initial organs damaged in SCD. The gold standard for assessment for splenic filtration function is a 99mTc sulfur-colloid liver spleen (LS) scan but it requires radiation. Pitted erythrocyte (PIT) and Howell–Jolly Bodies (HJBs) have been assessed as a better non-invasive biomarker for spleen function (Rogers et al., 2011).

6.7. Pitted RBC counts

Pitted RBCs are normally removed by healthy spleen. However, in the first year of their lives among SCD patients, more than 3.5% RBCs have pits, which is a sign of splenic dysfunction. Higher percentage of pitted RBCs before reaching the age of one year is a clinical indicator of unfavorable outcomes in the future (Miller et al., 2000). Hence, high numbers of pitted RBC counts may implicate splenic impairment. The procedure of pitted RBCs count is slow and laborious, requiring fixed RBCs to be analyzed under phase-contrast microscopy.

6.8. HJBs by flow cytometry

The presence of HJBs has been associated with hyposplenism. Measuring the levels of HJBs is a quick way of testing spleen function, and it is strongly linked with pitted RBC counts. This assay is currently offered by very few laboratories (Rogers et al., 2011).

6.9. Renal dysfunction

Kidney damage is the unavoidable outcome of SCD. Biomarkers for renal function in SCD are well established. The most routinely used biomarkers are blood urea nitrogen, creatinine concentration, urine albumin to creatinine ratio, etc. Renal

dysfunction shows up from early age in SCD patients, beginning with glomerulus hyperfiltration and ending in kidney failure in 30% of patients (De Franceschi et al., 2011). Micro albuminuria is common in childhood but it may continue to exist in 20% of adults with SCD in the form of nephritic range protein loss, with more than 3.5 g protein urea in 24 h (Scheinman, 2009). Asymptomatic hematuria is considered as one of the most prevalent features of sickle cell nephropathy. Prior to confirming the diagnosis of sickle cell nephropathy, other causes of renal dysfunction should be ruled out (58).

6.10. Pulmonary dysfunction

Major pulmonary dysfunction in children with SCD comprises of acute chest syndrome and bronchial hyperactivity (Eastman, 1984). The biomarkers associated with this condition are increased white blood cell counts (Maitre et al., 2000) and raised secretary phospholipase A2 levels (Styles et al., 1996).

7. Conclusion

SCD is characterized by a wide variety of abnormal levels of biomarkers that have been identified and associated with different pathological conditions. With the exception of a few, none of the biomarkers provide prognostic information regarding the management of the disease. Currently, measuring the hemoglobin values, HbF levels, reticulocytes counts and WBC counts are the primary diagnostic tools for the management of SCD. Extensive clinical trials are required to identify unambiguous independent markers for the corresponding pathological conditions of SCD. Existing information about biomarkers in SCD is still in its incipient stage and the future holds many challenges (Eastman, 1984).

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