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Genetic Modifiers of Sickle Cell Disease

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Abstract

Sickle cell anemia is associated with unusual clinical heterogeneity for a Mendelian disorder. Fetal hemoglobin concentration and coincident ∝ thalassemia, both which directly affect the sickle erythrocyte, are the major modulators of the phenotype of disease. Understanding the genetics underlying the heritable subphenotypes of sickle cell anemia would be prognostically useful, could inform personalized therapeutics, and might help the discovery of new "druggable" pathophysiologic targets. Genotype-phenotype association studies have been used to identify novel genetic modifiers. In the future, whole genome sequencing with its promise of discovering hitherto unsuspected variants could add to our understanding of the genetic modifiers of this disease.

Introduction

Sickle hemoglobinopathies are a related group of common and rare hemoglobin genotypes where all affected individuals are either homozygotes for the sickle hemoglobin (HbS) mutation (*HBB*; glu(E)6val(A); G*A*G-G*T*G; rs334) or compound heterozygotes for the HbS and another globin gene mutation. Homozygosity for HbS, or sickle cell anemia, is the most common genotype. Compound heterozygotes for HbS and other hemoglobin variants, like HbC (*HBB* glu6lys; HbSC disease), or with one of the many forms of β thalassemia (HbS- β thalassemia), usually have milder disease than HbS homozygotes because of the reduced intracellular concentration of HbS.[1] Vasoocclusion and hemolytic anemia are the major features of this Mendelian disease (for reviews see[1]) that is notable for its clinical and hematologic variability.[2,3] Fetal hemoglobin (HbF) concentration and ∝ thalassemia are the major modifiers of disease, but are unlikely to be the only ones.[4-11] The clinical features of the different sickle hemoglobinopathies have been reviewed many times and will not be discussed further. In the following sections we first discuss the effects of HbF and coincident α thalassemia on the subphenotypes of sickle cell anemia and conclude with emerging work on other genetic modifiers.

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To conserve space we avoid citing primary references that have appeared many times before. Instead, we cite review articles, especially when discussing well established background information that has been reviewed previously.

HbF

HbS polymerization is the major driver of sickle cell disease pathophysiology, and HbF the most important modulator of the clinical and hematologic features of this disease because it is unable to enter the HbS polymer and also reduces mean corpuscular HbS concentration.[12] Both the genetic basis of hemoglobin switching–the process by which the fetal globin genes (*HBG2* and *HBG1* or *HBG*) are silenced and adult globin genes (*HBB, HBD*) are expressed–and the effects of HbF in sickle cell anemia have been recently reviewed.[13,14]

Compound heterozygotes for HbS and gene deletion hereditary persistence of HbF (HPFH) have HbF concentrations of about 20% in each sickle erythrocyte. Carriers of this genotype are asymptomatic with only minor and clinically insignificant hematologic findings.[15,16] HbS-HPFH provides the best evidence that achieving high concentrations of HbF in most sickle erythrocytes can prevent clinically significant HbS polymerization and therefor a therapeutic goal to be vigorously pursued.

Effects of HbF on disease complications—Sickle cell anemia has many complications due to sickle vasoocclusion and hemolytic anemia and HbF affects the rate of some complications more than others.[17] Table 1 summarizes the relationships HbF concentration with the common complications of disease. High HbF is strongly associated with a reduced rate of acute painful episodes, fewer leg ulcers, and longevity. Less conclusive evidence supports an association of HbF with priapism, renal functional impairment, cerebrovascular disease and perhaps sickle vasculopathy as estimated by tricuspid regurgitant velocity. Studies at variance with these observations and disparities among study results might be a consequence of the diligence and methods of subphenotype ascertainment, ethnic and age differences amongst patients, variability of sample sizes and analytical approaches. For example, a retrospective study of Jamaicans with steady-state HbF levels below 1% compared them with patients having HbF levels 2.5%-3.4% and 4.6%-5.2%. As expected, packed cell volume and mean corpuscular volume increased with increasing HbF, but differences in the incidence of painful episodes and the acute chest syndrome were not apparent.[18] Perhaps comparisons of cases with more widely separated levels HbF would have changed these results as the methods used in this study to measure very low HbF concentrations can be error prone. In another study of African Americans with an average age of 19.2 years and mean HbF of 12±7%, HbF was not associated with painful events, acute chest syndrome or survival although there was a higher risk of stroke in patients with lower levels of HbF.[19] The reported HbF levels were much higher than those from other studies of a similarly aged population and the microchromatographic methods used to measure HbF differed from those used in most studies. Later reports by the same authors partially contravened their first conclusions.[20] It is difficult to reconcile these 2 reports with the wealth of biologic, laboratory and clinical data associating high HbF with a protective effect for many of the complications of sickle cell disease (Table 1).

Population differences in HbF and the genetic basis of HbF regulation—HbF levels are heritable.[21-23] The normal switch from fetal to adult hemoglobin is completed 1 to 2 months postnatally.[24] Still, some normal erythroid precursors continue to express *HBG*.[25] In sickle cell anemia the switch from fetal to adult globin is delayed and HbF levels are

usually increased throughout life. Three well established quantitative trait loci (QTL), one cis to the *HBB* gene-like cluster and two in trans, are the major known modulators of *HBG* expression. Others must exist but are either rare or restricted to selected populations with sickle cell anemia.

Cis-acting regulation of HbF: The HbS mutation is present on 5 different *HBB* gene-like cluster haplotypes that originated in Africa, the Middle East and India (reviewed in[26]). From studying populations representing these haplotypes it has been shown that HbF levels among carriers of the 4 most common haplotypes vary as follows: Arab-Indian (AI)>Senegal>Benin>Bantu. Within any haplotype group there is considerable variation in HbF level. An autochthonous origin of HbS in the Middle East and India is present on an AI *HBB*-like globin gene cluster haplotype. Sickle cell anemia and HbS-β^0 thalassemia in patients with the AI haplotype have a higher HbF concentration than comparable patients of African origin where the HbS gene is found with Benin, Bantu and Senegal haplotypes, although few HbS-β^0 thalassemia cases with a Senegal haplotype have been studied.[26-31] AI haplotype patients have mild, albeit not asymptomatic disease with frequent splenomegaly and osteonecrosis.[32] The AI haplotype is characterized by the presence of a 5′ *HBG2* Xmn1 C-T restriction site (rs7482144), a Hinc2 restriction site 5′ to *HBE1* (rs3834466) and insertion-deletion polymorphisms in other elements cis to *HBB.* In 132 untreated patients with sickle cell anemia and the AI haplotype, the HbF level averaged 17% (range, 4%-32%).[28,29,33] In African Americans, most of whom are compound heterozygotes or homozygotes for Benin and Bantu haplotypes that do not contain rs7482144, a marker of the Senegal haplotype, HbF levels are 5% to 8%.[34,3513] A recent study examined African Americans with unusually high HbF concentrations.[36] Compared with low HbF cases, they had significantly higher minor allele frequencies of the 2 known trans-acting elements associated with high HbF (see below), *BCL11A* and the intergenic interval between *HBS1L* and *MYB* (HMIP; 6q22-23). Individuals from the Saudi Southwestern Province with either sickle cell anemia or HbS-β^0 thalassemia had HbF levels lower that the Eastern Province patients yet higher than African American sickle cell patients.[37] Southwestern Province patients have the HbS gene on African *HBB* haplotypes, usually the Benin type; in contrast to African Americans, they rarely have priapism or leg ulcers.[38]

Reduced expression of *HBB*, as in the case of HbS-β^0 thalassemia, and cis-acting elementsthat minimize *HBG* silencing might also influence HbF. Among the cis-acting elements that could act synergistically to enhance *HBG* expression are unknown loci tagged by rs7482144, elements within the *HBD-HBG1* intergenic region, the *HBB* locus control region (LCR) HS-2 core and AT repeats ~530 bp 5′ to *HBB.* A similar putative mechanism of increased *HBG* expression combined with *HBB* suppression was described in Corfu $\delta^0\beta^+$ thalassemia where HbF is very high in homozygotes.[39] A candidate within this region is a 3.5 kb element near the 5’ portion of *HBD.* The *HBG* silencer BCL11A binds within this region that also has GATA-1 and HDAC1 sites[40] and its deletion delayed *HBG* to *HBB* switching. A high HbF phenotype might also be conditional on the presence of hemolysis causing erythroid marrow expansion as in carriers of the Corfu deletion and in HbS with the AI haplotype, HbF levels are normal.

In a genome-wide association study (GWAS) of black patients with sickle cell anemia using a discovery set of 848 cases and a replication set of 305 cases, with additional studies in Thai and Chinese individuals with β-thalassemia trait, a region on chromosome 11 containing olfactory receptor genes *OR51B5* and *OR51B6* was identified.[41] Elements within the olfactory receptor gene cluster might play a regulatory role in *HBG* expression.[42]

The Senegal haplotype is also marked by rs7482144. Homozygous and heterozygous carriers of this haplotype have high $^{G}\gamma$-globin levels compared with other African-origin haplotypes and might have higher HbF.[31] Eighty-seven kb within the *HBB* gene-like cluster were sequenced in patients of African origin and rs1012856 was in linkage disequilibrium (LD) with rs7482144 and more strongly associated with HbF than rs7482144. This SNP had an effect on HbF independent of rs7482144; rs7482144 had no effect on HbF independent of rs10128556.[43] Additionally, the association of olfactory receptor genes with HbF were not significant after conditioning in this SNP. The functional elements linked to the *HBB* gene-like cluster and tagged by these rs10128566 and other SNPs are unknown.

Trans-acting elements modulating HbF: Carriers of any HbS haplotype have considerable variance in HbF levels suggesting the importance of trans-acting QTL that modulate *HBG* expression. Two QTL in trans to *HBG* are HMIP and *BCL11A* (2p16.1). Polymorphisms in HMIP were associated with F-cell levels, accounted for 19.4% of the F-cell variance in normal Europeans and were distributed in 3 LD blocks. Overexpression of *MYB* in K562 cells inhibited *HBG* expression.[44] Low levels of *MYB* were associated with reduced cell expansion and accelerated erythroid differentiation, suggesting that variation in the intrinsic levels of *MYB* might affect HbF by its effect on the cell cycle. Rare *MYB* variants were associated with HbF.[43] Among individuals with 1 of 3 rare missense variants in *MYB*, HbF was 7.5% compared with 6.1% in cases without such a variant. Overexpression of microRNA-15a and -16-1 down-regulated *MYB* in $CD34^{+}$ erythroid progenitors and increased HbF.[45]

The most significant motif accounting for HMIP modulation of HbF is a 3 bp deletion polymorphism in complete LD with SNP rs9399137. This SNP was shown in several GWAS to be highly associated with HbF in multiple populations.[46-49] It is located near the erythroid-specific DNase I hypersensitive site 2 within the HMIP block 2, 42.6 kb upstream of *HBS1L* and 83.8 kb upstream of *MYB.* In close proximity to this deletion polymorphism are binding sites for TAL1, E47, GATA2, and RUNX1, all erythropoiesis-related transcription factors. Furthermore, the short DNA fragment encompassing the 3 bp deletion polymorphism appears to have enhancer-like activity based on in vitro transient transfection experiments.[47] The HMIP polymorphism is also associated with HbF among sickle cell anemia patients of African descent [41,48,50,51] though less significantly when compared with Europeans or Chinese due to a much lower minor allele frequency.[47] It is rare in AI haplotype Saudi patients and therefore not associated with HbF in this group (Bae et al, unpublished data). Other HMIP variants might be not tracked well by SNP rs9399137 so the role this locus plays in certain populations is still unknown.[43]

The consistent agreement of the HMIP association results across multiple populations where its minor allele frequency is high, in conjunction with erythropoiesis-related transcription

factor binding studies, phylogenetic conservation, and in vitro enhancer-like activity suggests that in many, but not all populations, the 3 bp deletion polymorphism is probably the most significant functional variant within this region accounting for its association with HbF level. In one study, expression of *HBS1L* was associated with HbF.[52]

The singular successes of GWAS in sickle cell anemia was the seminal and serendipitous discovery of *BCL11A* as a major regulator of hemoglobin gene switching and HbF.[53] This discovery was possible because of an experimental design that dichotomized the very highest (>95th percentile) and very lowest (<5th percentile) F-cell levels in a very small number of individuals and the large effect of this locus on HbF. In addition to *BCL11A* GWAS in sickle cell anemia have found the upstream olfactory receptor gene cluster[41] and a SNP on chr17p13.3, *GLP2R*, a glucagon-like peptide 2 receptor expressed in the gut to be associated with HbF. In a sex-stratified analysis, one intronic SNP (rs12103880) in *GLP2R* was associated with F-cells only in males.[54] Curiously, other GWAS using much larger numbers of patients did not find this gene associated with HbF. It is unclear why this association was only seen in males–higher HbF in females is presumably an X-linked function–and other HbF GWAS studies have adjusted for gender.[41] Perhaps F-cells are a different phenotype than HbF, although they are highly correlated[55], and F-cells were used as a phenotype in other GWAS.[47,53] More likely, the association of *GLP2R* with HbF is a false-positive result.

BCL11A, a developmentally regulated zinc finger protein gene and silencer of *HBG* expression was strongly associated with HbF concentrations in normal individuals and several different populations of patients with β thalassemia and with sickle cell anemia including AI haplotype Saudi patients (Bae H et al, personal communication). By its effects on HbF concentration, *BCL11A* modified the features of both diseases.[41,50,53,56,57] Binding sites for BCL11A have been described in HS-3 of the LCR and the Aγ-δ intragenic region using chromosome immunoprecipitation assays, and in a GGCCGG motif in the proximal promoter of *HBG*. Polymorphism within the 14 kb intron 2 of *BCL11A* correlated with HbF-cell numbers in several different populations. Individual variants and haplotypes at this locus accounted for up to 18% of HbF variance sickle cell anemia.[58] By studying ethnically distinct populations with sickle cell disease and β thalassemia it was suggested that possible functional motifs responsible for modulating HbF level or F-cell numbers might reside within or immediately adjacent to a 3 kb region bounded by rs1427407 (position 60,629,694) and rs4671393 (position 60,632,602) in intron 2 of *BCL11A*.[53,56,57]

Complexes of BCL11A with other proteins might mediate the suppressive effects of *BCL11A* on *HBG* expression. *KLF1*, an activator of *BCL11A*, plays an important role in hemoglobin switching and selected polymorphisms have been associated with the HPFH phenotype.[59,60] Associations of SNPs in *KLF1* with HbF in sickle cell anemia have not yet been reported but “functional” SNPs seem rare in AI haplotype patients and African Americans with very high HbF (Bae et al, Personal communication). SNPs in QTLs on chr11p16.1, chr2p16 and 6q22-23 explain about one third to one half of HbF variation in sickle cell anemia leaving much of the variance in HbF level unexplained. Other variants are likely to explain this “missing” heritability but are difficult to detect using GWAS, which in the case of sickle cell disease have examined relatively small samples.

HbF response to hydroxyurea—Hydroxyurea reduces the morbidity and mortality of sickle cell anemia, an effect mediated primarily, although probably not exclusively by its induction of HbF.[61-63] The HbF response to hydroxyurea is variable and some patients do not respond to treatment. Like baseline HbF levels, the HbF response to hydroxyurea is also heritable.[64] Changes of HbF induced by hydroxyurea can be substantial. In subjects who start with baseline HbF values between 5% and 10% increases can be 2 to 3 fold; subjects with very low baseline HbF can have 10-fold increases. These observations suggest that genetic modifiers could have large effects and be discoverable even with limited sample sizes.

The data available on the genetic basis of HbF response to hydroxyurea are not definitive and have yet to be replicated. In one study, 320 tag SNPs in 29 candidate loci within the 6q22.3–q23.2, 8q11–q12 and Xp22.2–p22.3 linkage peaks, in genes involved in the metabolism of hydroxyurea and in genes related to erythroid progenitor proliferation were studied in 137 sickle cell anemia patients. This work was done prior to the discovery of the association of *BCL11A* with *HBG* expression. SNPs in genes within the 6q and 8q linkage peaks, and also the *ARG2*, *FLT1*, *HAO2* and *NOS1* genes were associated with the HbF response to hydroxyurea.[65]

The failure of HbF to modulate uniformly all complications of sickle cell disease might be related to the heterogeneous distribution of HbF among sickle erythrocytes at both the baseline state and in response to hydroxyurea treatment and the premature destruction of erythrocytes that contain little HbF.[13] Many epidemiological studies suggested that disease complications most closely linked to sickle vasoocclusion and blood viscosity were robustly related to HbF concentration while complications associated with the intensity of hemolysis were less affected (reviewed in[17,66]). The weak or absent association of HbF with osteonecrosis, as exemplified in Saudi patients, suggests that increased blood viscosity associated with improved red cell survival due to high HbF and ∝ thalassemia that is common in in the Saudi population might dominate the reduction in HbS polymerization tendency.

∝Thalassemia

More than 30% of most populations with sickle cell anemia carry one or more determinants for ∝ thalassemia. In people of African descent this is usually heterozygosity or homozygosity for the $^{-3.7}$∝-globin gene deletion. α Thalassemia modulates sickle cell anemia by reducing the intracellular concentration of HbS that in turn decreases HbS polymer-induced cellular damage, which ameliorates hemolysis.[4]

The hematologic and laboratory changes in sickle cell anemia-∝ thalassemia include: higher hemoglobin concentration, lower MCV, higher HbA_2, lower reticulocyte count, lower bilirubin level, lower LDH, fewer dense and irreversibly sickled cells, increased erythrocyte lifespan; HbF concentration changed little. The magnitude of these changes is related to the number of deleted ∝-globin genes.[4]

Table 2 summarizes the effects of ∝ thalassemia on the common subphenotypes of sickle cell anemia. Heterogeneity among populations of patients with sickle cell anemia, the study

of small patient samples, inhomogeneity of the cohorts that sometimes include individuals with HbSC disease and HbS-β^+ thalassemia, and age differences among subjects in different studies have resulted in some reports that diverge from the majority conclusions that are cited in this table. As $\propto$ thalassemia is an important determinant of hemolysis, its presence is usually associated with fewer complications, like stroke, priapism and leg ulcers that have been closely associated with hemolysis.[67] Paradoxically, patients with sickle cell anemia-$\propto$ thalassemia do not have a reduction and might have an increase in complications like painful episodes, acute chest syndrome or osteonecrosis, and this has been ascribed to increased blood viscosity that results from the higher PCV in sickle cell anemia patients with $\propto$ thalassemia.

Other genetic modifiers

Discovering genetic modifiers of disease depends in part on the heritability of the trait. The stroke subphenotype of sickle cell anemia is heritable and therefore genetically modifiable.[68] Other subphenotypes, for example, acute chest syndrome and painful episodes, although likely to have some genetic basis [69,70] are more obviously influenced by environmental factors. It was suggested that the environment can change the epigenome and modulate gene expression without changing the genetic code.[11,71,72]

Genotype-phenotype association studies compare the odds of a selected subphenotype occurring in carriers of genetic variant compared with non-carriers. Alternatively, when the phenotype is quantifiable–HbF for example–the totality of data in a sample can be used for analysis, an advantage in a rare disease where accumulating sufficient patient samples for a case-control or dichotomized analysis is difficult. The prerequisites for any genetic association study are evidence that the phenotype examined is heritable and reproducible, a clear distinction between “cases” and “controls,” or sufficient variability of the quantitative trait, adequate patient numbers allowing robust statistical analysis and replication in groups of similar genetic ancestry, In the past 10 years both candidate gene association studies and GWAS been completed for many subphenotypes of sickle cell anemia. Almost uniformly the functional SNP or a putative mechanism whereby the disease is modified is unknown.

Candidate Gene Association Studies—Most candidate gene association studies were characterized by small sample sizes and no requirement to replicate the findings in other studies. This approach often lead to contradictory results when studies are compared. Table 3 provides a reasonably complete summary of candidate gene associations with common subphenotypes of sickle cell anemia. The following discussion focuses on the disease subphenotypes most rigorously studied and on results or polymorphisms that have been replicated.

Stroke, trans-cranial Doppler flow velocity, silent cerebral infarction: Non-hemorrhagic stroke is clinically important, can be definitively ascertained and, notwithstanding issues of large vs. small vessel disease, silent vs. clinical stroke and relative rarity compared with some other subphenotypes, has been the subphenotype most closely examined (Table 3). Using the resources of a study of hydroxyurea for secondary stroke prevention ((SWiTCH, #NCT00122980), 38 SNPs in 22 genes were genotyped in 130 well-documented stroke

patients and 103 non-stroke children with sickle cell anemia.[73] These SNPs were chosen based on previous candidate gene studies cited in Table 3. In addition to the known association of ∝ thalassemia with a reduced risk of stroke, 4 of the 38 SNPs tested were significantly associated with stroke risk, all with the same effect on stroke as previously published. *ANXA2* (rs11853426),*TEK* (rs489347), and *TGFBR3* (rs284875) variants were associated with increased stroke risk while *ADCY9* (rs2238432) was associated with decreased stroke risk. These observations confirmed prior work using Bayesian network modeling that tested 108 SNPs in 39 candidate genes and found a network of 31 SNPs in 12 genes that modulated the risk of stroke.[74] Although most other SNPs associated with stroke in the studies cited in Table 3 were tested, none could be replicated. Stroke is a complex trait, unlikely to be modulated by a single gene. Validated results of genetic associations might make it possible to add genetic tests to predictive modeling and improve the selection of patients for preventive treatments that have intrinsic liabilities, like chronic transfusion and hydroxyurea.

The TGF-β/Smad/BMP pathway: One of the surprises of candidate gene association studies is the consistent association of SNPs in the TGF-β (transforming growth factor-β) / Smad/BMP (bone morphogenetic protein) pathway with multiple subphenotypes of disease reported by 3 groups of independent investigators who studied different patient populations. In some instances the same SNP in the same gene has been associated with the same subphenotype. These associations are summarized in Tables 2 and 3 of reference.[10]

The TGF-β /Smad/BMP signaling pathway regulates diverse cellular processes. It signals through membrane-bound receptors, downstream Smad proteins and other signaling mediators and plays roles in inflammation, fibrosis, cell proliferation and hematopoiesis, osteogenesis, angiogenesis, nephropathy, wound healing and the immune response. The many complications of sickle cell anemia are effected by most of these processes so it is not unreasonable to suspect that perturbations of this pathway would modulate their development, progression and resolution.

GWAS—Thousands of disease-causing genes have been identified in Mendelian disorders by studying well characterized phenotypes and by using gene mapping techniques. However the same approach has not been as successful in identifying the genetic modifiers of common multigenic diseases like hypertension, diabetes, cardiovascular disease and dementia that do not follow Mendelian laws of inheritance. Sickle cell anemia is a classical Mendelian disorder but with a multigenic phenotype. Candidate gene-based association studies cannot find hitherto unsuspected novel genetic regions. GWAS have propelled us toward this goal but in rare diseases have progressed slowly because of the large sample sizes required to detect associations with small effect sizes that meet the stringent significance levels needed when hundreds of thousands of comparisons are being made. The role of GWAS in complex traits, using examples from work in sickle cell anemia, has been reviewed and discussed the issues of studying rare diseases.[75]

Studies of quantitative phenotypes other than HbF are just beginning to appear. Serum bilirubin levels have been associated with dinucleotide repeat polymorphisms in the *UGT1A1* promoter in normal populations and in patients with sickle cell anemia (Table 3).

When hemolysis occurs circulating heme increases, leading to elevated unconjugated bilirubin levels and an increased incidence of cholelithiasis. In a GWAS of bilirubin levels and cholelithiasis risk in a discovery cohort of 1,117 sickle cell anemia patients, 15 SNPs were associated with total bilirubin levels at the genome-wide significance level (5×10^{-8}). SNPs in *UGT1A1, UGT1A3, UGT1A6, UGT1A8* and *UGT1A10* were identified (most significant rs887829, $p = 9.08\times10^{-25}$). All of these associations were validated in 4 independent sets of more than 3,000 sickle cell anemia patients. A significant association was also noted when these SNPs were tested for their association with cholelithiasis (most significant p value 1.15×10^{-4}). These results confirm that the *UGT1A* region is the major regulator of bilirubin metabolism in African Americans with sickle cell anemia, similar to what is observed in other ethnicities.[76] In this analysis there was no association between *UGT1A1* SNPs and LDH, hemoglobin concentration and reticulocyte count.

In work published in abstract form (Milton JN, et al. Clinical and genetic variability of red blood cell hemolysis in sickle cell disease. Blood 2011;118.) a hemolytic score derived by principal component analysis[77] was heritable (Fig. 1A, B) and used as a subphenotype in a GWAS (Fig. 1C). The top SNP associated with hemolysis ($p=6.04\times10^{-07}$) was in *NPRL3* (rs7203560; chr16p13.3) a gene a gene harboring the major $\propto$-globin gene regulatory loci, HS-33, HS-40 and HS-48, within its other introns.[78] Rs7203560 is in perfect LD with rs2238368 and in strong LD with rs 2541612 ($D'=0.89$) and rs13331107 ($D'=0.61$) in the HS-33 to HS-40. When adjusted for HbF and $\propto$ thalassemia the association with *NPRL3* was weaker but still significant. A significant association between the hemolytic score and HbF was present after adjusting for age, sex, and $\propto$ thalassemia. Although both HbF and $\propto$ thalassemia are the major determinants of hemolysis in sickle cell anemia the hemolytic score was independently associated with rs7203560 in *NPRL3*. The functional basis for this association is unknown but the LD pattern within this region suggests that variation in the major $\propto$-globin gene regulatory loci could play a role.

TRV is likely to be a heritable trait, is a mortality risk factor in sickle cell disease and can be used as a marker of sickle vasculopathy.[79] In a GWAS published in abstract form (Bae H, et al. An elevated tricuspid regurgitant jet velocity in sickle cell disease is associated with polymorphisms in genes impacting innate immunity Blood 2011;118:514a.), 4 SNPs in *CSMD1* (CUB and Sushi multiple domains 1, a gene that inhibits the classical pathway of complement activation and complement-mediated hemolysis in sheep erythrocytes[80] were associated with TRV in 340 patients and replicated in 56 independent cases. No SNP reached genome-wide significance. Inflammation is a mediator of vascular biology in sickle cell disease and also a known modulator of idiopathic pulmonary hypertension. *CSMD1* mutations may alter the immune response.

Median lifespan for patients with sickle cell anemia in the United States was in the fifth decade.[81] To predict mortality in sickle cell disease a Bayesian network modeled 24 clinical events and laboratory tests to estimate disease severity represented by a score that predicted near-term mortality. The reliability of the model was supported by analysis of 2 independent patient groups.[82] In 1,265 patients with either “severe” or “mild” disease based on this network model of disease severity, a GWAS discovered 40 SNPs that were strongly associated with sickle cell severity (odds for association >1,000); of the 32 SNPs that could

be analyzed in an independent set of 163 patients, 5 replicated, 8 showed consistent effects although failed to reach statistical significance, whereas 19 did not show any convincing association. Among the replicated associations are SNPs in *KCNK6* a potassium channel gene. Using an analytical method that examined genetic regions, 27 genes with a strong enrichment of significant SNPs ($P < 10^{-6}$) were present and 20 were replicated with varying degrees of confidence. Among the novel genes identified by this analysis was the telomere length regulator gene *TNKS*.[83] These studies were the first to use GWAS to understand the genetic diversity that accounts the phenotypic heterogeneity of sickle cell anemia as estimated by an integrated model of severity. Both genetics and environment affect longevity, and although clearly a heritable trait in other populations[84], survival in sickle cell anemia is likely to be driven by the adverse effects of the disease rather than "longevity genes." Given the widespread use of hydroxyurea and its major effect on morbidity, mortality and laboratory tests in sickle cell anemia–the original network model used data that antedated the clinical use of hydroxyurea–it is unlikely that an analysis of the genetic associations with "untreated" sickle cell anemia in developed countries can be repeated.

Conclusions

Many of the subphenotypes of sickle cell anemia are heritable and likely to be influenced by networks of interacting genes but also by the environment. Genetic polymorphisms that affect the course of sickle cell anemia and its clinical and laboratory subphenotypes are potentially useful as prognostic markers, could guide personalized therapeutics and might suggest new "druggable" targets. The next phase of genetic association studies will be the application of whole genome sequencing with its promise of discovering new variants that point to novel disease-impacting pathways. For success in this quest, rigorous subphenotyping, careful case selection, avoiding sequencing errors, validation of new candidates in large populations and functional and mechanistic studies will be critical.

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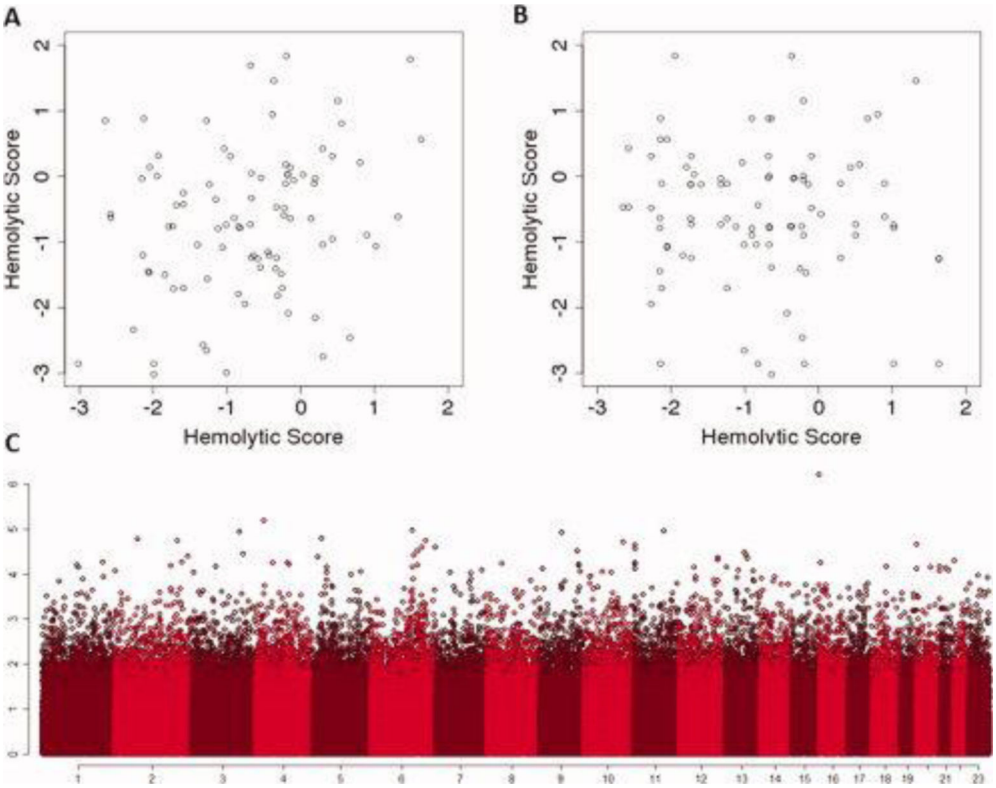

Genetic modifiers of sickle cell disease

Heritability of hemolytic score. The scatter plot in panel (A) shows hemolytic score of sib pairs ($r = 0.24$, $p = 0.02$) while panel (B) shows HbF of pairs of unrelated subjects ($r = 0.001$, $p = 0.52$). (C) Manhattan plot summarizing the results of GWAS of hemolytic score. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table 1

Relationship of HbF to common clinical and hematologic features of sickle cell disease.

Disease Sub-phenotype	Effects of HbF	References
Survival	High HbF prolongs survival in most untreated and hydroxyurea-treated cases	62,81,85-87
Painful episodes/dactylitis	High HbF reduces incidence	88,89
Acute chest syndrome	High HbF reduces rate	89,90
Leg ulcers	High HbF protective	91-93
Osteonecrosis	Equivocal evidence for a protective effect	32,94-98
Priapism	Little or no evidence of a protective effect	99,100
Renal function/albuminuria	Little or no evidence of a protective effect	101-106
Stroke, increased trans cranial Doppler velocity/ silent infarction	Equivocal or no evidence of a protective effect in infants; some evidence of protection in adults	107-111
Splenic sequestration/splenic function	Low HbF increases risk of sequestration and is associated with earlier loss of function. High HbF protective.	32,89,112
Bacteremia	Little or no evidence of a protective effect	113
Cholelithiasis	High HbF protective	97,114
Retinopathy	Low HbF possibly increases capillary occlusion	115
Sickle vasculopathy/TRJ velocity	Little or no evidence of a protective effect	79,116-119
Pregnancy/perinatal death	Decreased risk	120
Erythrocyte survival	High MC(HbF)C increases RBC lifespan	121
Hemoglobin level	High HbF associated with increased level	122

For nearly every disease subphenotype it is possible to find contradicting evidence. The studies cited contain the largest sample size and the most rigorous experimental design but no attempt was made to be exhaustive. For most subphenotypes, both children and adults are included. Portions of the Table were derived from.[123]

Table 2

Relationship of ∝ thalassemia to common clinical features of sickle cell disease.

Disease Sub-phenotype	Effects of ∝ thalassemia	References
Overall severity	Probably little effect	5
Stroke, silent infarction, TCD velocity	Reduces risk	73,74,110,124-126
Painful episodes	Increases risk	124,127
Acute chest syndrome	Reduces risk	124
Bacteremia	No effect	124
Osteonecrosis	Increases risk	128
Priapism	Reduces risk	99
Leg ulcers	Reduces risk	91
Sickle vasculopathy/TRJ velocity	Equivocal	129
Splenic sequestration/function	Reduces risk	130
Cholelithiasis	Reduces risk	131,132
Renal function/albuminuria/glomerular hyperfiltration	Reduces risk	106,133-135
Retinopathy	Possibly reduces capillary occlusion	136

(Modified from 5 with additional references)

Table 3

Candidate genes associated with subphenotypes of sickle cell anemia.

Disease Sub-phenotype	Genes involved and effect	References
Survival	Multiple including *TGFBR3*	137
Stroke, silent infarction, TCD velocity	Multiple gene identified, *VCAM1, ILR4, ADBR2, HLA, LDLR*, but few have been validated (see text)	73,74,138,139
Painful episodes	*GCH1*-results reported in abstract only. Biologically plausible. *MBL2*-in children, low expression associated with increased pain	70,140-142
Acute chest syndrome	Many genes have been "identified" but no study has been validated.	69,94,143,144
Bacteremia/Infection	*MBL2*-contradictory evidence in different populations that that low level protective. Other genes include *CCL5*, various HLA alleles, *IGF1R*, TGF-β/SMAD/BMP pathway	113,145,146
Osteonecrosis	Little evidence for *MTHFR; BMP6*-results validated in 2 different populations	94,147-149
Priapism	*KL, TEK, TGFBR3, AQP1*	150-152
Leg ulcers	TGF-β/SMAD/BMP pathway, *KL*, possibly HLA alleles	91,152,153
Sickle vasculopathy/TRJ velocity	*BMP6, TGFBR3, ACVR1, BMP2*	154
Cholelithiasis	Promoter repeats in *UGT1A1* associated with serum bilirubin	155,156
Renal function/albuminuria/glomerular hyperfiltration	*DARC* FY- associated with proteinuria, TGF-β/Smad/BMP pathway, *MYH9, APOL1*	157-159
Multiple subphenotypes	Duffy antigen receptor (*DARC*) No relationship to leg ulcers, nephropathy, priapism, osteonecrosis, response to opioids	160,161