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The Genetics of Hemoglobin A_2 Regulation in Sickle Cell Anemia

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Abstract

Hemoglobin A_2, a tetramer of α- and δ-globin chains, comprises less than 3% of total hemoglobin in normal adults. In northern Europeans, single nucleotide polymorphisms (SNPs) in the *HBS1L-MYB* locus on chromosome 6q and the *HBB* cluster on chromosome 11p were associated with HbA_2 levels. We examined the genetic basis of HbA_2 variability in sickle cell anemia using genome-wide association studies (GWAS). HbA_2 levels were associated with SNPs in the *HBS1L-MYB* interval that affect erythropoiesis and HbF expression and SNPs in *BCL11A* that regulate the γ-globin genes. These effects are mediated by the association of these loci with γ-globin gene expression and fetal hemoglobin (HbF) levels. The association of polymorphisms downstream of

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the β-globin gene (*HBB*) cluster on chromosome 11 with HbA_2 was not mediated by HbF. In sickle cell anemia, levels of HbA_2 appear to be modulated by trans-acting genes that affect *HBG* expression and perhaps also elements within the β-globin gene cluster. HbA_2 is expressed pancellularly and can inhibit HbS polymerization. It remains to be seen if genetic regulators of HbA_2 can be exploited for therapeutic purposes.

Introduction

Hemoglobin A_2 (HbA_2: $\alpha_2\delta_2$), a tetramer of α- and δ-globin chains, forms less than 3% of total hemoglobin in normal adults.[1] It has no known physiological function, but elevated levels are associated with β-thalassemia trait. Its levels are heritable. Genetic variation explains 42% of total HbA_2 variability using a variance components model.[2] The genetic basis of HbA_2 regulation has been studied in northern Europeans where single nucleotide polymorphisms (SNPs) in the *HBS1L-MYB* locus on chromosome 6q and the *HBB* cluster on chromosome 11p were associated with HbA_2. This effect is presumably a result of modifying the kinetics of erythropoiesis and transcription within the *HBB* gene cluster.[2]

HbA_2 can inhibit the polymerization of sickle hemoglobin (HbS) and it has a pancellular distribution.[3-6] High HbA_2 might therefore be of benefit in sickle cell anemia.[7] Polymorphisms of the *HBS1L-MYB* interval that are associated with hematopoiesis and fetal hemoglobin (HbF) concentration are differentially distributed among populations,[8] and higher than normal HbF levels are characteristic of sickle cell anemia.[9] Stress erythropoiesis might also be partly responsible for increased HbF levels. A reciprocal relationship between HbA_2 and HbF levels is present in acquired disorders where HbF levels are increased [10] and HbA_2 levels are lowest in cells with increased HbF and in individuals with high HbF.[11, 12] Menzel found that females with more F-cells had lower HbA_2 than males with fewer F-cells.[2] To confirm and extend the observations on the genetic regulation of HbA_2 we examined the genetic basis of HbA_2 variability in sickle cell anemia.

Materials & methods

Study subjects

The discovery cohort included 618 unrelated African American subjects from the Cooperative Study of Sickle Cell Disease (CSSCD; NCT00005277).[13] To replicate the associations with $p<1E-5$ we used 128 African American patients from the Pulmonary Hypertension and Sickle Cell Disease with Sildenafil Therapy (Walk-PHaSST;NCT00492531)[14], 45 African American cases from the Pulmonary Hypertension and the Hypoxic Response in Sickle Cell Disease (PUSH; NCT 00495638) [15], and 580 Chinese subjects from the Hong Kong β-thalassemia trait study (Table 1).[16] The studies were approved by the Institutional Review Boards of all participating sites.

Genotyping

The DNA from the CSSCD, Walk-PHaSST, PUSH and Hong Kong samples were genotyped using the Illumina Human610-Quad array and processed and analyzed as described.[17] We used the genome-wide identity by descent analysis in PLINK to discover

unknown relatedness. Pairs with identity by descent measurements greater than 0.2 were deemed to be related subjects and only one subject was included in the analysis. We analyzed only common SNPS (MAF>0.05), from individuals with call rates of at least 98%.

Phenotype

HbA_2 was measured by DEAE Cellulose column chromatography [18, 19] (CSSCD) or high performance liquid chromatography (Walk-PHaSST, PUSH, β-thalassemia cohort). HbF was measured by alkali denaturation [20] (CSSCD) and HPLC (Walk-PHaSST, PUSH, β-thalassemia cohort). Measurements taken when a subject was under 5 years old were discarded as HbA_2 values may not be stable before this age. HbA_2 values of below 1.4 or above 7.9 were discarded in the sickle cell anemia cohorts, as values outside of these bounds are considered to be biologically unlikely and due to instrumentation or recording errors.

The CSSCD and Walk-PHaSST were both longitudinal studies, and some subjects had multiple HbA_2 and HbF measurements and median HbA_2, HbF and age measurements were used. In the CSSCD HbA_2 and HbF were measured on the same blood sample.

Statistical analysis and genome-wide association study

Data are represented as mean and standard deviation or median and range. Relation between HbA_2 and MCV was tested using linear regression, and the effect of α thalassemia was tested using an interaction between MCV and α thalassemia. Association between HbA_2 and each SNP was tested using an additive genetic model in PLINK, [21] and a normal distribution for HbA_2. The normal distribution assumption is shown by the QQ-plot (Figure S1). Two models were conducted, (1) adjusted for age and sex; (2) adjusted for age, sex, and HbF. SNPs that reached a significant association with HbA_2 with p<1E-5 in the CSSCD samples were assessed in the remaining cohorts. At this significance level, if no associations were present and all SNPs were independent, we would expect to observe 5.5 false positives. The presence of gene deletion α thalassemia was adjusted for in the CSSCD cohort. A meta-analyses using Metal software assessed all studies simultaneously.[22]

For replication of the 9 available SNPs from the 13 previously found associated with HbA_2 in normal individuals[2], we used a p-value threshold of p<0.05/9. To further examine previously reported associations, we conducted a mediation analysis to determine if there was mediation in the SNP/HbA_2 association by HbF.[23] Mediation was assessed by performing a series of regressions adjusted for age and sex:

$$HbA_2 = i_1 + c\,(SNP) + e_1 \quad (1)$$

$$HbA_2 = i_2 + c'\,(SNP) + b\,(HbF) + e_2 \quad (2)$$

$$HbF = I_3 + a\,(SNP) + e_3 \quad (3)$$

If, for a SNP, *c* and *a* are significantly different from zero, these regressions are then used to evaluate the effect of SNP on HbA_2 through HbF by (c-c') (Figure S2).[23] Values shown in

Table 1 and Supplement Table 3 correspond to hypothesis tests of whether this mediated effect is nonzero.

Results

Patient Characteristics

CSSCD and PUSH patients were younger than Walk-PHaSST (Table S1).[24] The distribution of HbA $_2$ in all cohorts is shown in Figure S3 A-D. The mean value of HbA $_2$ was ~ 3% In the CSSCD, 3.8% in Walk-PHaSST and 3.5% in PUSH reflecting higher HbA_2 measurement by HPLC in the presence of HbS. The Hong Kong cohort with β-thalassemia trait had a mean of 5.4%. In this cohort, HbA_2 levels are also affected by the nature of the β-thalassemia mutation that also is associated with the numbers of F-cells.[16]

HbA_2 and HbF were correlated (r=-0.20, nonzero with p=3.7E-24; Figure 1) as were HbA_2 and MCV (r=0.58, p=1.8E-137) and to a lesser extent HbF and MCV (r=0.14, p=1.7E-08). The relationship between HbA_2 and MCV remained significant after adjusting for HbF (p<2E-16). α Thalassemia was associated with a reduction of MCV; (Figure S4). Increased HbA_2 in sickle cell anemia-α thalassemia is a result α/δ-globin dimerizing more readily than α/β^S-globin.[18] α Thalassemia modified the relationship between HbA_2 and MCV: without α thalassemia, a regression of HbA_2 on MCV yielded a slope of -0.028; with α thalassemia, the slope was -0.038 (a -33% change, p= 0.0339).(Table S2.)

Replication of association in northern Europeans

In northern Europeans the *HSB1L-MYB* intergenic region on chromosome 6q and the *HBB* cluster on chromosome 11p were strongly associated with HbA_2.[2] Of 13 reported associations, 9 SNPs were available for our analysis (Table 1). When adjusted for age and sex, nominal replication (p<0.05) was obtained for 4 SNPs in the *HSB1L-MYB* intergenic region (bold font in Table 1), and 1 SNP in the *HBB* gene cluster. Considering only SNPs with p<0.05 in the analysis adjusted for age, sex and HbF, only rs9494145 in *HSB1L-MYB* (p=4.5E-02), rs11036212 in *HBB* (p=4.5E-02), and rs11036364 in HBB (p=**4.64E-03)** were replicated. As reported, R^2 between 0.01 and 0.02 for their top SNPs, we have power between 0.70 and 0.94 to nominally replicate (p=0.05) these results if such an association exists. For these SNPs in the CSSCD, we obtained R^2 between 0.00017 and 0.0067.

The association between HbA_2 and the SNPs identified in [2] were tested for mediation by HbF in CSSCD samples. Only rs9494145 in chromosome 6q was associated with HbA_2 and this effect was mediated by HbF (p=6.33E-05). The effect of rs11036212 in chromosome 11p was not mediated by HbF. This is consistent with Menzel's results the results[2] as their associations in chromosome 11 remained strong even after adjustment for HbF.

GWAS results in CSSCD

GWAS in the CSSCD are shown in Figure 2, Table 2 and Supplement Tables 3 and 4. Rs6038123 with the smallest p-value adjusted for age, sex, and HbF (p=1.37E-06) (20p12.3; position 5, 291,506), is located downstream of *UBE2D3P1* (ubiquitin-conjugating enzyme E2D 3 pseudogene 1). This SNP also had the largest partial R^2 (0.039) for any SNPs

analyzed in the CSSCD samples. Uda et al found an association of rs6037828 (20p13; position 508,365) with HbF in Sardinians with β thalassemia.[25] In a meta-analysis of more than 2000 patients with sickle cell anemia we found no association of any SNP on chromosome 20 with HbF at p<1E-5.[26] The next-strongest association, adjusted for HbF, is at rs822278, located in the intergenic between *MRPL49P2* (mitochondrial ribosomal protein L49 pseudogene 2) and *FGF20* (fibroblast growth factor 20) on chromosome 8 (p=1.4E-06). These genes have not been implicated in hematological traits, and replication is required to ensure they are not false positives.

Replication in secondary cohorts was attempted for all CSSCD variants with p<1E-5 (Table S3, age and sex adjusted, and Table S4, age, sex, and HbF adjusted). Nominal replication (p<0.05) was achieved for rs766432 and rs10195871 in *BCL11A.* Both SNPs are associated with HbF levels.[17, 27-33] Rs766432 achieved genome-wide significance in meta-analysis (adjusted for age and sex only), with p=3.03E-10. All SNPs in Tables S3 and S4 reached statistical significance after Bonferroni correction (p < 0.05/14=0.004), and several SNPs had also consistent effects in the different studies. Mediation analysis suggested that the association of HbA_2 with *BCL11A* is at least partially mediated by HbF.

Discussion

Normal newborns have less than 0.3% HbA_2.[34] We studied the genetic regulation of HbA_2 in sickle cell anemia and confirmed in part associations previously reported in normal northern Europeans.[2] A mediation analysis suggested, as did the studies of Menzel et al, that SNPs associated with HbA_2 in the *HBS1L-MYB* locus on chromosome 6q affect HbA_2 levels partially through their effects on HbF. An association of SNPs in *BCL11A* with HbA_2, is also mediated by the effects of this gene on HbF expression. The enhancer of *MYB* within HMIP-2 of the *HBS1LMYB* locus [8, 35-37] is likely to modulate HbF by dual effects on erythropoiesis and the activation of other genes like *KLF1* that modulate HbF expression. [37] Low MYB levels accelerate erythroid differentiation leading to release of early progenitors synthesizing predominantly HbF and also directly affect γ-globin gene expression.[38, 39]

The association of SNPs within the *HBB c*luster and HbA_2 levels was not mediated by HbF. SNPs previously associated with HbA_2 [2], and the SNP we replicated (rs11036212), are in the 3’ hypersensitive site (HS; downstream) of the *HBB* cluster, whereas SNPs associated with HbF are 5’ (upstream) of *HBB*. We could not replicate the associations found in Sardinians with β-thalassemia trait where the associations were in the 5’ β-globin gene locus control region.[26] Our results explain only a small amount of the variance of HbA_2 that is likely to be affected by the complex regulation of globin gene expression.[40]

HbF is the major modulator of the phenotype of sickle cell anemia, but HbA_2 has similar effects on HbS polymerization.[9, 41] Whether *HBD* expression can be increased, perhaps by targeting the mutated CACC binding site for KLF1 in the *HBD* promoter[7], is unknown. Other differences in *HBD* might thwart this approach.[42-49] Pancellular expression of HbA_2 predicts that increased HbA_2 levels could be especially beneficial.[50]

Gene expression within the *HBB* gene cluster is partially a function of competition among proximal gene promoters for transcription factors and the β-globin locus control region.[51] This is exemplified by increases in HbF and HbA_2 when the promoter of the β-globin gene is deleted or mutated.[52-55] Substantial increases in HbA_2 and HbF was found in HbS-β^0 thalassemia when the β-globin gene promoter was deleted, and this was associated with mild disease.[56]

HbF was measured by two different methods our cohorts examined; within the range of HbF observed, both give similar results. Nevertheless, this could be a confounder.[57] HbA_2 was also measured by differed means. When done by HPLC (Walk-PHaSST and PUSH trials) HbA_2 levels are increased due to the co-elution of some HbS adducts.[58]

In sickle cell anemia, levels of HbA_2 appear to be modulated by genes that directly and indirectly effect *HBG* expression and perhaps also by regulation within the β-globin gene cluster. It remains to be seen if these observations can be exploited for therapeutic purposes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Steinberg MH, Adams JG III. Hemoglobin A2 : Origin, evolution, and aftermath. Blood. 1991; 78:2165–2177. [PubMed: 1932737]
2. Menzel S, Garner C, Rooks H, et al. HbA2 levels in normal adults are influenced by two distinct genetic mechanisms. Br J Haematol. 2013; 160:101–105. [PubMed: 23043469]
3. Heller P, Yakulis V. The distribution of hemoglobin A2. Ann NY Acad Sci. 1968; 165:54. [PubMed: 4187025]
4. Poillon WN, Kim BC, Rodgers GP, et al. Sparing effect of hemoglobin F and hemoglobin A 2 on the polymerization of hemoglobin S at physiologic ligand saturations. Proc Natl Acad Sci USA. 1993; 90:5039–5043. [PubMed: 7685112]
5. Nagel RL, Bookchin RM, Labie D, et al. Structural basis for the inhibitory effects of hemoglobin F and hemoglobin A2 on the polymerization of hemoglobin S. Proc Natl Acad Sci USA. 1979; 76:670–672. [PubMed: 284392]
6. Waterman MR, Cottam GL, Shibata K. Inhibitory effect of deoxyhemoglobin A2 on the rate of deoxyhemoglobin S polymerization. J Mol Biol. 1979; 129:337–341. [PubMed: 480348]
7. Zhu J, Chin K, Aerbajinai W, et al. Recombinant erythroid Kruppel-like factor fused to GATA1 up-regulates delta- and gamma-globin expression in erythroid cells. Blood. 2011; 117:3045–3052. [PubMed: 21220744]
8. Farrell JJ, Sherva RM, Chen ZY, et al. A 3-bp deletion in the HBS1L-MYB intergenic region on chromosome 6q23 is associated with HbF expression. Blood. 2011; 117:4935–4945. [PubMed: 21385855]
9. Akinsheye I, Alsultan A, Solovieff N, et al. Fetal hemoglobin in sickle cell anemia. Blood. 2011; 118:19–27. [PubMed: 21490337]

10. Dover GJ, Boyer SH, Zinkham WH, et al. Changing erythrocyte populations in juvenile chronic myelocytic leukemia: Evidence for disordered regulation. Blood. 1977; 49:355–365. [PubMed: 264791]
11. Weatherall, DJ.; Clegg, JB. The Thalassaemia Syndromes. Blackwell; Oxford: 1981.
12. Henri A, Testa U, Tonthat H, et al. Disappearance of Hb F and i antigen during the first year of life. Am J Hematol. 1980; 9:161–170. [PubMed: 6159787]
13. Gaston M, Rosse WF. The cooperative study of sickle cell disease: review of study design and objectives. Am J Pediat Hematol-Oncol. 1982; 4:197–201.
14. Machado RF, Barst RJ, Yovetich NA, et al. Hospitalization for pain in patients with sickle cell disease treated with sildenafil for elevated TRV and low exercise capacity. Blood. 2011; 118:855–864. [PubMed: 21527519]
15. Dham N, Ensing G, Minniti C, et al. Prospective echocardiography assessment of pulmonary hypertension and its potential etiologies in children with sickle cell disease. Am J Cardiol. 2009; 104:713–720. [PubMed: 19699350]
16. Gibney GT, Panhuysen CI, So JC, et al. Variation and heritability of Hb F and F-cells among beta-thalassemia heterozygotes in Hong Kong. Am J Hematol. 2008; 83:458–464. [PubMed: 18266208]
17. Solovieff N, Milton JN, Hartley SW, et al. Fetal hemoglobin in sickle cell anemia: genome-wide association studies suggest a regulatory region in the 5′ olfactory receptor gene cluster. Blood. 2010; 115:1815–1822. [PubMed: 20018918]
18. Steinberg MH, Rosenstock W, Coleman MB, et al. Effects of thalassemia and microcytosis upon the hematological and vaso- occlusive severity of sickle cell anemia. Blood. 1984; 63:1353–1360. [PubMed: 6722353]
19. Huisman, THJ. Human hemoglobins.. In: Yunis, JJ., editor. Biochemical Methods in Red Cell Genetics. Academic Press; Orlando: 1969. p. 391-504.
20. Betke K, Marti HR, Schlicht I. Estimation of small percentages of foetal haemoglobin. Nature. 1959; 184:1887–1888. [PubMed: 13821046]
21. Purcell S, Neale B, Todd-Brown K, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet. 2007; 81:559–575. [PubMed: 17701901]
22. Willer CJ, Li Y, Abecasis GR. METAL: fast and efficient meta-analysis of genomewide association scans. Bioinformatics. 2010; 26:2190–2191. [PubMed: 20616382]
23. MacKinnon DP, Fairchild AJ, Fritz MS. Mediation analysis. Annu Rev Psychol. 2007; 58:593–614. [PubMed: 16968208]
24. Milton JN, Gordeuk VR, Taylor JGt, et al. Prediction of fetal hemoglobin in sickle cell anemia using an ensemble of genetic risk prediction models. Circ Cardiovasc Genet. 2014; 7:110–115. [PubMed: 24585758]
25. Uda M, Galanello R, Sanna S, et al. Genome-wide association study shows BCL11A associated with persistent fetal hemoglobin and amelioration of the phenotype of beta-thalassemia. Proc Natl Acad Sci USA. 2008; 105:1620–1625. [PubMed: 18245381]
26. Bae H, Baldwin CT, Sebastiani P, et al. Meta-analysis of 2040 sickle cell anemia patients: BCL11A and HMIP are the major genetic modifiers of HbF in African Americans. Blood. 2012; 120:1961–1962. [PubMed: 22936743]
27. Menzel S, Garner C, Gut I, et al. A QTL influencing F cell production maps to a gene encoding a zinc-finger protein on chromosome 2p15. Nat Genet. 2007; 39:1197–1199. [PubMed: 17767159]
28. Galarneau G, Palmer CD, Sankaran VG, et al. Fine-mapping at three loci known to affect fetal hemoglobin levels explains additional genetic variation. Nat Genet. 2010; 42:1049–1051. [PubMed: 21057501]
29. Lettre G, Sankaran VG, Bezerra MA, et al. DNA polymorphisms at the BCL11A, HBS1L MYB, and beta-globin loci associate with fetal hemoglobin levels and pain crises in sickle cell disease. Proc Natl Acad Sci U SA. 2008; 105:11869–11874.
30. Sankaran VG, Menne TF, Xu J, et al. Human Fetal Hemoglobin Expression Is Regulated by the Developmental Stage-Specific Repressor BCL11A. Science. 2008; 322:1839–1842. [PubMed: 19056937]
31. Sankaran VG, Xu J, Byron R, et al. A functional element necessary for fetal hemoglobin silencing. N Engl J Med. 2011; 365:807–814. [PubMed: 21879898]

32. Bauer DE, Kamran SC, Lessard S, et al. An erythroid enhancer of BCL11A subject to genetic variation determines fetal hemoglobin level. Science. 2013; 342:253–257. [PubMed: 24115442]
33. Sedgewick A, Timofeev N, Sebastiani P, et al. BCL11A (2p16) is a major HbF quantitative trait locus in three different populations. Blood Cells, Mol Dis. 2008; 41:255–258. [PubMed: 18691915]
34. Felicetti L, Novelletto A, Benincasa A, et al. The HbA/HbA2 ratio in newborns and its correlation with fetal maturity. Br J Haematol. 1984; 56:465–471. [PubMed: 6199038]
35. Menzel S, Jiang J, Silver N, et al. The HBS1L-MYB intergenic region on chromosome 6q23.3 influences erythrocyte, platelet, and monocyte counts in humans. Blood. 2007; 110:3624–3626. [PubMed: 17712044]
36. Thein SL, Menzel S, Peng X, et al. Intergenic variants of HBS1L-MYB are responsible for a major quantitative trait locus on chromosome 6q23 influencing fetal hemoglobin levels in adults. Proc Natl Acad Sci USA. 2007; 104:11346–11351. [PubMed: 17592125]
37. Stadhouders R, Aktuna S, Thongjuea S, et al. HBS1L-MYB intergenic variants modulate fetal hemoglobin via long-range MYB enhancers. JClin Invest. 2014; 124:1699–1710. [PubMed: 24614105]
38. Stamatoyannopoulos G. Control of globin gene expression during development and erythroid differentiation. Exp Hematol. 2005; 33:259–271. [PubMed: 15730849]
39. Tallack MR, Perkins AC. Three fingers on the switch: Kruppel-like factor 1 regulation of gamma-globin to beta-globin gene switching. Curr Opin Hematol. 2013; 20:193–200. [PubMed: 23474875]
40. Sankaran VG, Xu J, Orkin SH. Advances in the understanding of haemoglobin switching. Br J Haematol. 2010; 149:181–194. [PubMed: 20201948]
41. Steinberg MH, Sebastiani P. Genetic modifiers of sickle cell disease. Am J Hematol. 2012; 87:824–826. [PubMed: 22641479]
42. Wood WG, Old JM, Roberts AVS, et al. Human globin gene expression control of beta, delta and beta delta chain production. Cell. 1978; 15:437–446. [PubMed: 719749]
43. Ross J, Pizarro A. Human beta and delta globin messenger RNA's turn over at different rates. J Mol Biol. 1983; 167:607–617. [PubMed: 6876159]
44. Proudfoot NJ, Shander MHM, Manley JL, et al. Structure and in vitro transcription of human globin genes. Science. 1980; 209:1329–1336. [PubMed: 6158093]
45. Humphries RK, Ley T, Turner P, et al. Differences in human alpha-, beta- and delta- globin gene expression in monkey kidney cells. Cell. 1982; 30:173–183. [PubMed: 6290076]
46. Kosche KA, Dobkin C, Bank A. DNA sequences regulating human beta globin gene expression. Nucl Acids Res. 1985; 13:7781–7793. [PubMed: 2999704]
47. Grosveld GC, Rosenthal A, Flavell RA. Sequence requirements for the transcription of the rabbit beta- globin gene in vivo: The -80 region. Nuc Acids Res. 1982; 10:4951–4971.
48. Tang DC, Ebb D, Hardison RC, et al. Restoration of the CCAAT box or insertion of the CACCC motif activates delta-globin gene expression. Blood. 1997; 90:421–427. [PubMed: 9207479]
49. Tang DC, Rodgers GP. Activation of the human delta-globin gene promoter in primary adult erythroid cells. Br J Haematol. 1998; 103:835–838. [PubMed: 9858241]
50. Steinberg MH, Chui DH, Dover GJ, et al. Fetal hemoglobin in sickle cell anemia: a glass half full? Blood. 2014; 123:481–485. [PubMed: 24222332]
51. Hanscombe O, Whyatt D, Fraser P, et al. Importance of globin gene order for correct developmental expression. Genes & Devel. 1991; 5:1387–1394. [PubMed: 1714415]
52. Codrington JF, Li HW, Kutlar F, et al. Observations on the levels of Hb A 2 in patients with different á-thalassemia mutations and a ë chain variant. Blood. 1990; 76:1246–1249. [PubMed: 1698102]
53. Patterson M, Walker L, Eng B, et al. High Hb A2 beta-thalassemia due to a 468 bp deletion in a patient with Hb S/beta-thalassemia. Hemoglobin. 2005; 29:293–295. [PubMed: 16370492]
54. Steinberg MH, Coleman MB, Adams JG III, et al. High hemoglobin A2 β-thalassemia. J Lab & Clin Med. 1991; 118:382–382. [PubMed: 1940581]

55. Donaldson A, Thomas P, Serjeant BE, et al. Foetal haemoglobin in homozygous sickle cell disease: a study of patients with low HbF levels. Clin Lab Haematol. 2001; 23:285–289. [PubMed: 11703409]

56. Waye JS, Chui DH, Eng B, et al. Hb S/beta zero-thalassemia due to the approximately 1 4-kb deletion is associated with a relatively mild phenotype. Am J Hematol. 1991; 38:108–112. [PubMed: 1719807]

57. Fabry, ME. Laboratory Diagnosis of hemoglobin Disorders and Animal Models for Their Study.. In: Steinberg, MH.; Forget, BG.; Higgs, DR., et al., editors. Disorders of Hemoglobin: Genetics, Pathophysiology, and Clinical Management. Cambridge University Press; Cambridge: 2001. p. 910-940.

58. Suh DD, Krauss JS, Bures K. Influence of hemoglobin S adducts on hemoglobin A2 quantification by HPLC. Clin Chem. 1996; 42:1113–1114. [PubMed: 8674201]

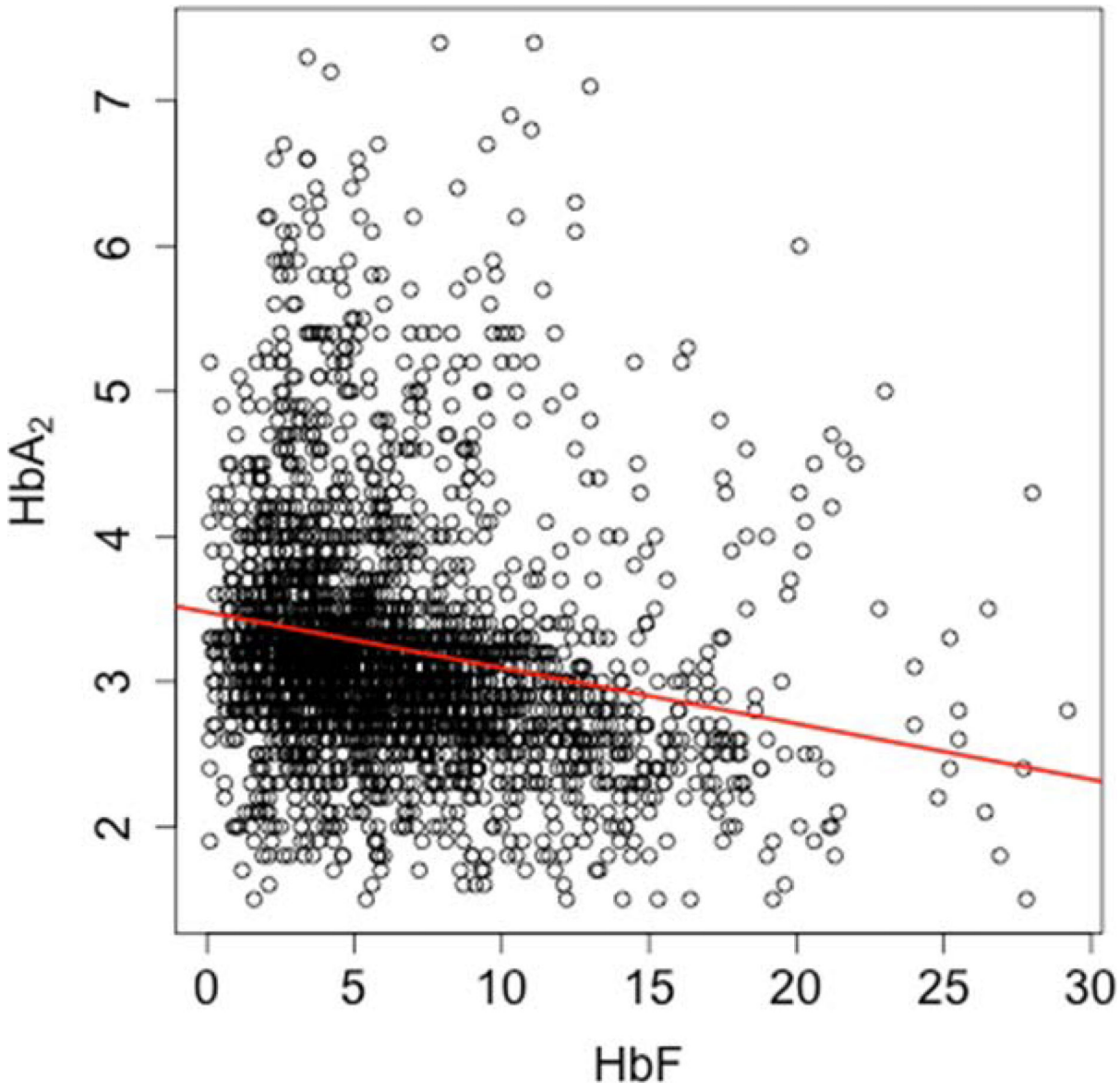

Figure 1.

Association between HbA_2 and HbF. HbA_2 and HbF are correlated (r=-0.20; nonzero with p=3.7E-24).

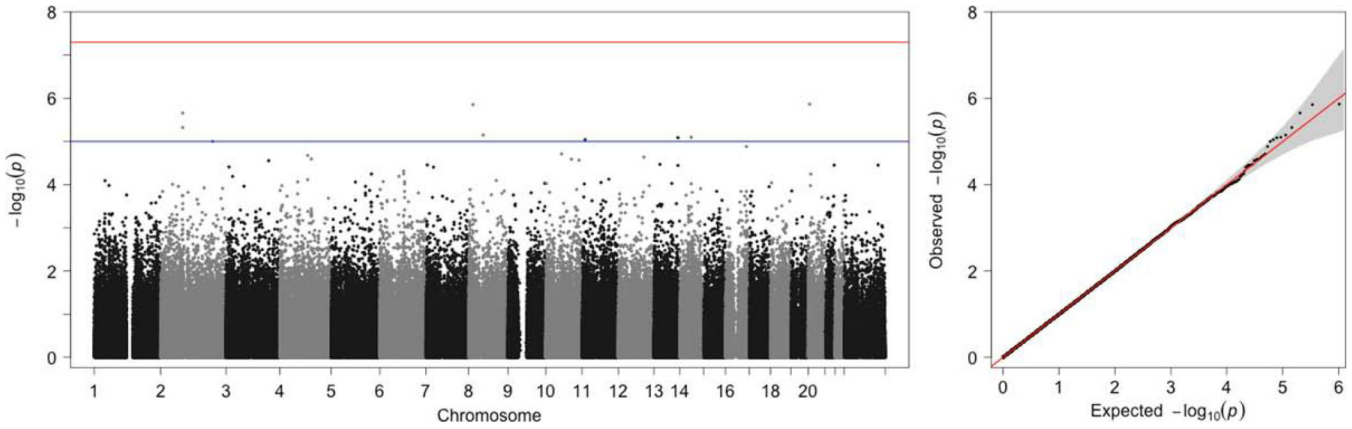

Figure 2.

Genome-wide association results for CSSCD adjusted for age, sex, and HbF. Left Panel: Manhattan plots of –log10(p-values) (y-axis) versus SNP positions (x-axis). No SNPs achieve genome-wide significance, but we find suggestive results on chromosomes 2, 6, and 11. Right Panel: QQ-plots for CSSCD GWAS results.

Table 1

Replication of results from (Menzel et al., 2013) [2] in the CSSCD cohort.

CHR	SNP	BP	Menzel P	P adj	CSSCD Effect*	P*	Effect adj**	P adj**	Effect HbF***	P HbF***	Effect Mediation+	P Mediation+
Chromosome 6q												
6	rs1547247	135432529	3.02E-08	1.39E-05	**−0.17**	**2.17E-02**	−0.12	1.07E-01	1.72	2.22E-03	*− 0.05*	*6.30E-03*
6	rs7775698	135460328	2.51E-09	3.30E-05	**−0.08**	**4.54E-02**	−0.06	1.54E-01	0.81	1.02E-02	*− 0.03*	*1.78E-02*
6	rs9399137	135460711	5.19E-09	4.50E-05	**−0.17**	**1.98E-02**	−0.08	2.66E-01	2.97	9.22E-08	*− 0.09*	*8.16E-05*
6	rs4895441	135468266	5.72E-09	5.50E-05	−0.09	1.25E-01	−0.03	5.46E-01	1.72	8.06E-05	*− 0.05*	*9.12E-04*
6	rs9376092	135468837	8.69E-09	7.16E-05	−0.03	4.71E-01	−0.02	7.07E-01	0.46	1.42E-01	*− 0.01*	*1.52E-01*
6	rs9494145	135474245	1.03E-08	1.42E-05	**−0.22**	**9.81E-04**	**−0.13**	**4.10E-02**	2.78	1.81E-08	**−0.08**	**6.33E-05**
Chromosome 11p												
11	rs11036212	5178401	1.45E-09	1.03E-08	**−0.08**	**2.26E-02**	−0.07	4.50E-02	0.37	1.67E-01	*− 0.01*	*1.77E-01*
11	rs7950726	5182023	1.23E-11	1.99E-10								
11	rs12787404	5182342	6.82E-12	1.01E-10								
11	rs10837582	5185284	3.12E-08	7.23E-08	0.03	6.62E-01	0.02	7.23E-01	−0.21	7.01E-01	*0.01*	*7.02E-01*
11	rs12793110	5188141	5.11E-12	7.73E-11								
11	rs10837628	5200980	1.53E-09	2.41E-09	0.03	6.60E-01	0.02	7.46E-01	−0.28	6.05E-01	*0.01*	*6.06E-01*
11	rs11036364	5205580	2.80E-09	2.59E-08	**0.35**	**1.37E-02**	**0.39**	**4.64E-03**	1.22	2.52E-01	*− 0.04*	*2.60E-01*

Results shown are regression effect sizes and p-values for

Italicized values in the mediation analysis are for an analysis where either no statistically significant adjusted effect on HbA_2 or no statistically significant association with HbF was observed for that SNP. These values should not be interpreted for mediation, but are provided for completeness.

*HbA_2 adjusted for age and sex

**HbA_2 adjusted for age, sex, and HbF

***HbF adjusted for age and sex

+mediation analysis.

Table 2

Top associations between SNPs and HbA_2 in CSSCD GWAS.

					Adjusted for age and sex		Adjusted for age, sex, and HbF		Mediation by HbF
CHR	**SNP**	**BP**	**MAF**	**A1**	**Effect**	**P**	**Effect**	**P**	**P**
2	rs766432	60573474	0.29	C	−0.195	**8.10E-08**	−0.1326	4.98E-04	**3.50E-05**
2	rs10195871	60574093	0.33	A	−0.166	**1.69E-06**	−0.112	1.64E-03	**2.35E-05**
2	rs17020489	81675692	0.3	A	0.1787	**8.00E-07**	0.1668	**2.20E-06**	1.73E-01
2	rs17020632	81765823	0.31	C	0.1667	**4.17E-06**	0.161	**4.75E-06**	5.13E-01
2	rs1113932	192760598	0.06	G	0.2881	2.00E-05	0.2895	**9.95E-06**	9.31E-01
8	rs822278	16536220	0.47	G	0.1613	**8.89E-07**	0.1539	**1.40E-06**	3.49E-01
8	rs1425912	54241746	0.36	G	0.1572	**7.65E-06**	0.1532	**7.09E-06**	6.37E-01
10	rs10887055	123789677	0.26	G	0.1746	**2.51E-06**	0.1523	2.70E-05	**1.55E-02**
11	rs11021763	11273929	0.37	G	0.1474	2.39E-05	0.1502	**9.09E-06**	7.45E-01
13	rs1536807	40267790	0.44	A	−0.1514	**8.11E-06**	−0.1371	3.39E-05	8.19E-02
13	rs2940695	107365156	0.25	A	0.175	**5.24E-06**	0.1665	**8.19E-06**	3.61E-01
14	rs2296274	60986931	0.15	A	−0.2204	**3.49E-06**	−0.2063	**8.00E-06**	2.19E-01
16	rs11150150	77897032	0.34	G	−0.1627	**4.21E-06**	−0.1501	1.31E-05	1.39E-01
20	rs6038123	5220152	0.2	A	−0.2138	**4.69E-06**	−0.2193	**1.37E-06**	6.19E-01

SNPs significant at p<1E-05 in at least one analysis of CSSCD.