Fetal haemoglobin response to hydroxycarbamide treatment and sar1a promoter polymorphisms in sickle cell anaemia

Chutima Kumkaek,1 James G. Taylor VI,2 Jianqiong Zhu,1 Carolyn Hoppe,1 Gregory J. Kato2,4 and Griffin P. Rodgers1
1Molecular and Clinical Hematology Branch, NIDDK, NIH, Bethesda, MD, 2Pulmonary and Vascular Medicine Branch, NHLBI, NIH, Bethesda, MD, 3Department of Hematology/Oncology, Children’s Hospital and Research Center at Oakland, Oakland, CA, and 4Critical Care Medicine, Clinical Center, NIH, Bethesda, MD, USA

Received 21 November 2007; accepted for publication 5 December 2007
Correspondence: Griffin P. Rodgers, Molecular and Clinical Hematology Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, 10 Center Dr. Rm 9N115, Bethesda, MD 20892, USA.
E-mail: gr5n@nih.gov

Summary
The hydroxycarbamide (HC)-inducible small guanosine triphosphate (GTP)-binding protein, secretion-associated and RAS-related (SAR) protein has recently been shown to play a pivotal role in HBG induction and erythroid maturation by causing cell apoptosis and G1/S-phase arrest. Our preliminary analysis indicated that HC inducibility is transcriptionally regulated by elements within the SAR1A promoter. This study aimed to assess whether polymorphisms in the SAR1A promoter are associated with differences Hb F levels or HC therapeutic responses among sickle cell disease (SCD) patients. We studied 386 individuals with SCD comprised of 269 adults treated with or without HC and 117 newborns with SCD identified from a newborn screening program. Three previously unknown single nucleotide polymorphisms (SNPs) in the upstream 5’UTR (−809 C>T, −502 G>T and −385 C>A) were significantly associated with the fetal haemoglobin (HbF) response in Hb SS patients treated with HC (P < 0.05). In addition, four SNPs (rs2310991, −809 C>T, −385 C>A and rs4282891) were significantly associated with the change in absolute HbF after 2 years of treatment with HC. These data suggest that variation within SAR1A regulatory elements might contribute to inter-individual differences in regulation of HbF expression and patient responses to HC in SCD.

Keywords: hydroxycarbamide, SAR1A, sickle cell disease, fetal haemoglobin, single nucleotide polymorphism.
phosphatidylinositol 3 (PI3) kinase and extracellular protein-related kinase (ERK) phosphorylation with increased p21 and GATA-2 expression (Tang et al., 2005).

SARIA belongs to the small GTPase superfamily and encodes a GTP-binding protein SAR1a. This protein plays a key role in initializing transport from the endoplasmic reticulum (ER) to the Golgi apparatus. The localization of SARIA in the endoplasmic reticulum and its association with HBG expression demonstrated in our recent study suggest that SARIA also plays a special role in haemoglobin regulation (Tang et al., 2005). However, the precise pathway(s) through which SARIA regulates HBG remain unknown. SARIA may increase the transport of membrane-bound transcription factor precursors from the ER to the Golgi. The proteolytic cleavage of the precursor proteins in the Golgi activate cytosolic fragments that enter the nucleus and regulate erythroid-specific transcription factors, such as GATA, eventually modulating HBG expression. Moreover, previous studies have illuminated a pivotal role of the p38 mitogen-activated protein kinase (MAPK) pathway during GTP-mediated erythroid differentiation of K562 cells with the accumulation of HBG mRNA (Osti et al., 1997; Moosavi et al., 2007). Activators of the soluble guanylate cyclase (sGC) and protein kinase G (PKG) pathways have been implicated in the regulation of HBG expression in both erythroleukemic cells and in primary erythroblasts (Ikuta et al., 2001). Expression of HBG and the sGC alpha subunit are correlated, indicating that GTP-binding proteins may participate in HBG induction.

Preliminary data from our study indicated that HC inducibility is transcriptionally regulated and localized to elements in the SARIA promoter. Accordingly, we hypothesized that DNA sequence variation within the SARIA promoter might explain differences in individual responses to HC therapy. To test this hypothesis, we identified the single nucleotide polymorphism (SNPs) in the SARIA promoter by DNA sequencing and examined these variants in an association study of sickle cell anaemia patients treated with HC.

Materials and methods

Subjects

DNA samples and laboratory data were from unrelated individuals with SCD who enrolled in a Sickle Cell Pulmonary Hypertension Screening Study at the National Institutes of Health (NIH) and Howard University (ClinicalTrials.gov Identifier: NCT00011648). The study had enrolled 282 subjects as of December, 2005, of which 269 had sufficient clinical data for inclusion in the present study (Taylor et al., 2008). All subjects were at least 18 years of age and provided written informed consent for participation in this Institutional Review Board (IRB) approved protocol. Cases and controls were defined as adults with SCD who were treated with or without HC respectively. Additionally, 32 of these 269 subjects had quantitative high performance liquid chromatography (HPLC) HbF measurements and other laboratory values determined prior to and during HC therapy. The mean length of follow-up evaluation for patients with HbF measured at the end of the study was 21 months (maximum, 33 months; minimum, 8 months). DNA was also available from 117 subjects identified with SCD by a state newborn screening program during a single calendar year, where DNA was collected anonymously with prospective exemption from IRB review.

Polymerase chain reaction (PCR) and DNA sequencing

A 2265 base pair fragment which included the SARIA upstream promoter region, exon 1, and a portion of intron 1 was amplified using gene-specific primers: forward primer, 5' ATGTGCACAACAAATGCGCTGT 3'; reverse primer, 5' GAA-ACTGTTATCCGCCCCAG 3'. The PCR conditions were an initial denaturation at 95°C for 3 min, followed by 35 cycles at 95°C for 45 s, 56°C for 1 min and 2 min at 72°C. Finally an additional elongation step was carried out at 72°C for 7 min. The PCR products were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). Purified PCR products were directly sequenced in both directions by using Big Dye chemistry (Applied Biosystems, Foster City, CA, USA). The BioEdit and Clustal W programs were used to multiple align individual sequences with the reference sequence (GenBank accession number: NT008583 or March, 2006 assembly hg18: chr1:71599909-71602173).

Statistical analysis

Comparisons of genotype and allele frequencies between cases and controls were carried out using chi-squared tests of association. Three genetic models (dominant, codominant and recessive) for modulation of response to HC treatment were tested. Genotype specific risks were estimated as odds ratio (OR) with 95% confidence intervals (95% CI). Multiple logistic regression (JMP 6.0.3) was used to investigate the association between individual SNPs and the change in HbF level after HC treatment. Haplotypes were determined using Phase 2.1 (Stephens & Donnelly, 2003). Probability differences of P < 0.05 were considered as statistically significant without additional correction for multiple comparisons.

Results

Of 282 enrolled adults at NIH, 13 patients were excluded because of incomplete data. The majority was Hb SS (202 cases, 71.6%), 44 haemoglobin SC (15.6%), 16 sickle beta-thalassaemia (5.7%), 4 sickle beta-thalassaemia (1.4%), two haemoglobin SD (0.7%), one haemoglobin S0Arab (0.4%) and two with incompletely characterized sickle beta-thalassaemia (0.7%). The presence of coincident alpha-thalassaemia (alpha7 deletion) was also determined for 260 SCD subjects (92.2%, n = 282): 179 (68.8%) with alpha/alpha, 73 (28.1%) with alpha/alpha, seven (2.7%) with alpha-alpha and one (0.4%) with alpha-alpha. No
\(\alpha^2\) deletions were detected (89.4\%, \(n = 252\) out of total 282) (Taylor et al., 2008).

In order to determine the pattern of mutation and polymorphism across putative HC responsive regulatory elements in SCD patients, we designed sequence-specific primers corresponding to a 23 kb fragment of SARI\(A\) on chromosome 10 which was completely sequenced in all subjects. Twenty DNA sequence variants, including two insertion/deletion polymorphisms and nine that have been previously reported in dbSNP (http://www.ncbi.nlm.nih.gov/SNP/index.html), were identified among a total of 386 patients, consisting of 269 adults at NIH and 117 newborns with SCD. Fourteen variants were within the upstream promoter region, four SNPs were in the 5′UTR encoded within exon 1 (+14 T>A, rs3812693, +31 T>C and rs3812692), and 2 SNPs were within the first intron (rs4282891 and intron 1 + 140 C>G). Using Phastcons genomic sequence alignments from 17 vertebrate species including Homo sapiens (http://www.genome.ucsc.edu; May, 2004 hg17 assembly), we identified phylogenetically conserved blocks of sequence across this 23 kb region of SARI\(A\). Four SNPs (−30 C>G, +14 T>A, rs4282891 and intron 1 + 140 C>G) corresponded to highly conserved non-coding nucleotide sites (Phastcons scores > 0.4), which is strongly suggestive of an increased likelihood that these SNPs represent functional variants.

When SARI\(A\) sequence data were compared between NIH subjects and the newborn SCD cohort, differences in genotype frequency were observed for rs2310991 in the upstream promoter region [Odds ratio (OR) = 1.9, 95% CI = 1.1–3.2, \(P = 0.009\)] and +31 T>C in the 5′UTR [OR 95% CI 1.3–7.3; 95% CI; \(P < 0.001\)] (data not shown).

Individual SARI\(A\) variants were then analysed for association with HbF levels, expressed as percentage of total haemoglobin (%) or as absolute HbF (g/l), among 69 cases vs. 107 controls. Three SNPs in the upstream promoter region (−809 C>T, −502 G>T and −385 C>A) were variably associated with higher percentage HbF under a co-dominant genetic model even after statistical correction (Table I). The major allele frequency of −809 C>T, −502 G>T and −385 C>A were 0.93, 0.98 and 0.96 respectively. When the analysis was repeated using absolute HbF concentration as a clinical endpoint between HC cases and SS controls, only one of these three markers showed evidence for an association (SNP −502 G>T; data not shown). None of the 20 variants were associated with total haemoglobin levels as the outcome measure between cases and controls (data not shown).

In addition, 32 of the Hb SS subjects had prospective, regular clinical evaluations including quantitative HbF

### Table I. SNPs associated with a higher percent HbF (%HbF) with hydroxycarbamide treatment.

| SNP | rs Number | Ch 10 map position | Dominant (HbF %) | Codominant (HbF %) | Recessive (HbF %) | Major allele frequency |
|-----|------------|---------------------|------------------|---------------------|------------------|----------------------|
| −1377 (G>T)* | rs2310991 | 71601652 | 0.027† | 0.0218 | 0.1369 | 0.45 |
| −809 (C>T) | rs76901216 | 71601084 | 0.0013 | 0.0008† | ND | 0.93 |
| −743 (G>A)* | rs10999169 | 71601018 | 0.0418 | 0.0108 | 0.0079 | 0.77 |
| −605/−606 (→−T)* | rs11438971 | 71600680/71600879 | ND | ND | 0.0231 | 0.01 |
| −502 (G>T) | rs76901217 | 71600777 | 0.0300 | 2.1266E-07† | ND | 0.98 |
| −460 (C>G) | rs76901218 | 71600735 | 0.0084 | 0.0129 | ND | 0.98 |
| −432 (T→−)* | rs11284333 | 71600707 | 0.0199 | ND | 0.0319 | 0.61 |
| −420 (TTTT→−)* | rs10577419 | 71600699 | 0.0310 | ND | 0.017 | 0.60 |
| −417 (T→−)* | rs5785963 | 71600692 | 0.0258 | ND | 0.0140 | 0.61 |
| −396 (T>C) | rs76901219 | 71600671 | 0.0264 | ND | ND | 0.98 |
| −385 (C>A) | rs76901220 | 71600660 | 0.0344 | 1.0610E-07† | ND | 0.96 |
| −372 (G>A) | rs76901221 | 71600647 | 0.0161 | 0.0833 | 0.0027† | 0.89 |
| −45 (G>A) | rs76901222 | 71600320 | 0.0348 | 0.5382 | ND | 0.98 |
| −30 (C>G) | rs76901223 | 71600305 | 0.0264 | ND | ND | 1.00 |
| +14 (T>A)* | rs76901224 | 71600262 | 0.0288 | ND | ND | 1.00 |
| +27 (C>A)* | rs3812693 | 71600249 | 0.0146 | 0.0284 | ND | 0.98 |
| +31 (T>C)* | rs76901225 | 71600245 | 0.0159 | 0.6734 | ND | 0.95 |
| +68 (C>T)* | rs3812692 | 71600208 | 0.0228 | ND | ND | 0.99 |
| Intron 1 +100 (G>A)* | rs4282891 | 71600075 | 0.0193 | 0.8913 | ND | 0.98 |
| Intron 1 +140 (C>G) | rs76901226 | 71600035 | 0.0267 | ND | ND | 0.99 |

*Previously reported in dbSNP.
†Present within the 5′ UTR.
‡Significant \(P\)-value after Bonforni correction.

Bold: SNP with \(P\)-value \(\leq 0.05\).
ND, not determined.

Major allele frequency was calculated from genotype frequency of Hb SS patients.
measurements before and during 2 years of HC therapy. When this patient subset was analysed, only rs2310991 was significantly associated with the change in the percentage of HbF. However, four markers (rs2310991, −809 C>T, −385 C>A and rs4282891) were significantly associated with the change of absolute HbF concentration. The most statistically significant P-value for an association was at rs4282891 (P = 0.0012), which is phylogenetically conserved in vertebrates (Phastcons score = 0.5984) and present intron 1 of SAR1A (Table II).

Considering all 20 markers, there were 64 total haplotypes including seven common haplotypes (>5% frequency) spanning 1617 nucleotides. Significant individual associations observed for markers −809 C>T, −502 G>T, and −385 C>A in the overall population and the 32 prospective follow-up subjects reflected a co-dominant haplotypic effect for the TGA variant (n = 23, 4.6%) and CGC wild type (n = 447, 89.8%) SAR1A promoter haplotypes.

**Discussion**

HC therapy in sickle cell anaemia increases the circulating HbF concentration in most individuals, although a large proportion of compliant patients experience either a complete failure to respond or have a small, clinically insignificant increase in HbF (Ma et al, 2007). Even among patients who are identified as HC responders, the magnitude of HbF increase is variable (Charache et al, 1995; ; Steinberg et al, 1997; Zimmerman et al, 2004; Bakanay et al, 2005; Ma et al, 2007). Presently, the underlying reason for this variability is not known (Steinberg et al, 1997; Bakanay et al, 2005).

Our previous work identified a novel role of SAR1A in HBG induction that is distinct from its previously established protein-trafficking function. SAR1A participates in HBG expression and erythroid cell maturation after HC treatment by regulating PI3 kinase/ERK and GATA-2/p21-dependent signal transduction pathways (Tang et al, 2005). These results led us to hypothesize that functional polymorphisms in regulatory elements of HC inducible genes, like SAR1A, modulate HbF levels or HC therapeutic responses. The present study identified all of the common nucleotide variants across a 2.3 kb region, which includes the promoter and first exon of SAR1A, including a subset of markers that are associated with either differences in HbF levels or change in HbF levels in sickle cell anaemia patients treated with HC. Individual associations with higher HbF concentration in HC-treated patients were detectable under three different genetic models (Table I), in addition to a more rigorous logistic regression association analysis of a subset of prospectively treated patients (Table II). Similar to our present findings in SAR1A, all of the presently known SNP markers in HC inducible genes associated with higher HbF are located within either promoter regions or the first intron 1 of candidate genes (Ma et al, 2007). In the slightly larger population studied by Ma et al (2007), variants in genes like FLT1, ARG2, HAO2 and NOS1 influenced HbF treatment response. Collectively, these association studies suggest that a specific transcriptionally regulated
gene expression profile may be initiated by HC therapy and that genetic variants within regulatory elements of these genes could modulate this response.

Comparative sequence analysis and cross-species conservation have previously been used to identify putative genes and regulatory elements in small genomic regions, suggesting that this is a viable approach for hypothesis generation and fine mapping functional variants underling genetic association studies (Pennacchio et al., 2001; Woolfe et al., 2005). Conserved sites within a 2-3 kb region of SAR1A were identified using the University of California Santa Cruz phastCons algorithm, which is based on a two state phylogenetic hidden Markov model that determines a likelihood ratio for conservation at a single nucleotide site across vertebrates (Siepel et al., 2005). We identified four variants, including three previously unknown markers (−30 C>G, +14 T>A, rs4282891 and intron 1 + 140 C>G), occurring at highly conserved SAR1A nucleotides. Thus, these variants are priority candidates for additional study to evaluate their role in regulating SAR1A transcription, potentially such using novel methods as high throughput transcriptional profiling for cloned promoter haplotypes in response to therapeutic agents like HC (Idelman et al., 2007).

SAR1A joins an expanding list of candidate loci including the genes listed above and regions of chromosomes 6q, 8q and Xp that might modulate HbF expression or HC responses. In humans, there are two SAR genes; SAR1A on chromosome 10 and SAR1B on chromosome 5. Although, these paralogs share high degree of amino acid sequence homology (89%), their expression patterns are distinct. SAR1A is expressed ubiquitously, most abundantly in prostate, thyroid and adrenal gland while SAR1B has a tissue-specific pattern with moderate expression in heart, liver, skeletal muscle and testis (He et al., 2002; Tang et al., 2005). These differences in expression patterns suggest different biological roles in various tissues and involvement in different human disease processes. Indeed, SAR1B mutations cause lipid absorption disorders, such as chylomicron retention disease (CMRD; MIM 246700) and Anderson disease (MIM 607689). In contrast, SAR1A mutations were not identified in either of these syndromes (Jones et al., 2003). To date, there are no known human SAR1A human mutations although this association study suggests that some SNPs could subtly alter SAR1A expression or function.

Finally, others have also examined SAR1A polymorphisms and haemolysis-driven phenotypes of SCD, such as leg ulcers, but these studies have not demonstrated significant associations (Nolan et al., 2006). Similarly in this study, there was no association between SAR1A SNPs and pulmonary hypertension (e.g. tricuspid regurgitant jet velocity >2.5 m/s, data not shown), which is also believed to be a haemolysis-associated complication of SCD. However, it is worthwhile to note that the Nolan leg ulcer study evaluated SNPs (rs870801 in intron 1 and rs2271690 in intron 3) that are located approximately 10 kb pairs apart from one another within a 203 kb gene (SAR1A). While these negative results suggest an unlikely role for involvement of SAR1A in response to haemolysis in SCD, neither study utilized high density, haplotype tagged markers to interrogate this gene. Additional study of the role of this gene in response to HC therapy and haemolysis are warranted before firm conclusions can be made.

In conclusion, SAR1A polymorphisms might contribute to the regulation of HbF expression and modulate patient responses to HC in sickle cell anemia. However, replication in independent populations and targeted functional studies are needed to confirm the validity of these associations.

Acknowledgements

This work was supported by the Intramural Research Program of the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health. The authors thank the investigators of Sickle Cell Pulmonary Hypertension Screening Study who obtained blood samples for DNA-based studies. We also acknowledge Dr Suthat Fucharoen, Thalassemia Research Center, Institute of Science and Technology for Research and Development, Mahidol University for his helpful discussions and on-going support of the project.

References

Bakanay, S.M., Dainer, E., Clair, B., Adekile, A., Daitch, L., Wells, L., Holley, L., Smith, D. & Kutlar, A. (2005) Mortality in sickle cell patients on hydroxyurea therapy. Blood, 105, 545–547.

Charache, S., Terrin, M.J., Moore, R.D., Dover, G.J., Barton, F.B., Eckert, S.V., McMahon, R.P. & Bonds, D.R. (1995) Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. Investigators of the Multicenter Study of Hydroxyurea in Sickle Cell Anemia. New England Journal of Medicine, 332, 1317–1322.

Cokic, V.P., Smith, R.D., Beleslin-Cokic, B.B., Njoroge, J.M., Miller, J.L., Gladwin, M.T. & Schechter, A.N. (2003) Hydroxyurea induces fetal hemoglobin by the nitric oxide-dependent activation of soluble guanylyl cyclase. The Journal of Clinical Investigation, 111, 231–239.

Collins, F.S., Green, E.D., Guttmacher, A.E. & Guyer, M.S. (2003) A vision for the future of genomics research. Nature, 422, 835–847.

Fucharoen, S., Siritanaratkul, N., Winichagoon, P., Chowthaworn, J., Siriboon, W., Muangsup, W., Chaicharoen, S., Poolsup, N., Chin-davijak, B., Pootrakl, P., Piknjikjum, A., Schechter, A.N. & Rodgers, G.P. (1996) Hydroxyurea increases hemoglobin F levels and improves the effectiveness of erythropoiesis in beta-thalassemia/hemoglobin E disease. Blood, 87, 887–892.

He, H., Dai, F., Yu, L., She, X., Zhao, Y., Jiang, J., Chen, X. & Zhao, S. (2002) Identification and characterization of nine novel human small GTPases showing variable expressions in liver cancer tissues. Gene expression, 10, 231–242.

Idelman, G., Taylor, J.G., Tongbai, R., Chen, R.A., Haggerty, C.M., Bilke, S., Chanock, S.J. & Gardner, K. (2007) Functional profiling of uncommon VCAM1 promoter polymorphisms prevalent in African American populations. Human Mutation, 28, 824–829.

Ikuta, T., Ausenda, S. & Cappellini, M.D. (2001) Mechanism for fetal globin gene expression: role of the soluble guanylate cyclase-cGMP-dependent protein kinase pathway. Proceedings of the National Academy of Sciences of the United States of America, 98, 1847–1852.
Jones, B., Jones, E.L., Bonney, S.A., Patel, H.N., Mensenkamp, A.R., Eichenbaum-Voline, S., Rudling, M., Myrdal, U., Annesi, G., Naik, S., Meadows, N., Quattrone, A., Islam, S.A., Naoumova, R.P., Angelin, B., Infante, R., Levy, E., Roy, C.C., Freemont, P.S., Scott, J. & Shoulders, C.C. (2003) Mutations in a Sar1 GTPase of COPII vesicles are associated with lipid absorption disorders. Nature Genetics, 34, 29–31.

Ma, Q., Wyszynski, D.F., Farrell, J.J., Kutlar, A., Farrer, L.A., Baldwin, C.T. & Steinberg, M.H. (2007) Fetal hemoglobin in sickle cell anemia: genetic determinants of response to hydroxyurea. The Pharmacogenomics Journal, 7, 386–394.

Moosavi, M.A., Yazdanparast, R. & Lotfi, A. (2007) ERK1/2 inactivation and p38 MAPK-dependent caspase activation during guanosine 5’-triphosphate-mediated terminal erythroid differentiation of K562 cells. The International Journal of Biochemistry & Cell Biology, 39, 1685–1697.

Nolan, V.G., Adewoye, A., Baldwin, C., Wang, L., Ma, Q., Wyszynski, D.F., Farrell, J.J., Sebastiani, P., Farrer, L.A. & Steinberg, M.H. (2006) Sickle cell leg ulcers: associations with haemolysis and SNPs in Klotho, TEK and genes of the TGF-beta/BMP pathway. British Journal of Haematology, 133, 570–578.

Okpala, I.E. (2005) New therapies for sickle cell disease. Hematology/Oncology Clinics of North America, 19, 975–987.

Ostì, F., Corradini, F.G., Hanua, S., Matteuzzi, M. & Gambari, R. (1997) Human leukemia K562 cells: induction to erythroid differentiation by guanine, guanosine and guanine nucleotides. Haematologica, 82, 395–401.

Pennacchio, L.A., Olivier, M., Hubacek, J.A., Cohen, J.C., Cox, D.R., Fruchart, J.C., Krauss, R.M. & Rubin, E.M. (2001) An apolipoprotein influencing triglycerides in humans and mice revealed by comparative sequencing. Science, 294, 169–173.

Rodgers, G.P., Dover, G.J., Noguchi, C.T., Schechter, A.N. & Nienhuis, A.W. (1990) Hematologic responses of patients with sickle cell disease to treatment with hydroxyurea. New England Journal of Medicine, 322, 1037–1045.

Siepel, A., Bejerano, G., Pedersen, J.S., Hinrichs, A.S., Hou, M., Rosenbloom, K., Clawson, H., Spieth, J., Hillier, L.W., Richards, S., Weistock, G.M., Wilson, R.K., Gibbs, R.A., Kent, W.J., Miller, W. & Haussler, D. (2005) Evolutionarily conserved elements in vertebrate, insect, worm, and yeast genomes. Genome Research, 15, 1034–1050.

Steinberg, M.H. (2005) Predicting clinical severity in sickle cell anemia. British Journal of Haematology, 129, 465–481.

Steinberg, M.H., Lu, Z.H., Barton, F.B., Terrin, M.L., Charache, S. & Dover, G.J. (1997) Fetal hemoglobin in sickle cell anemia: determinants of response to hydroxyurea. Multicenter Study of Hydroxyurea. Blood, 89, 1078–1088.

Stephens, M. & Donnelly, P.A. (2003) A comparison of Bayesian methods for haplotype reconstruction from population genotype data. The American Journal of Human Genetics, 73, 1162–1169.

Tang, D.C., Zhu, J., Liu, W., Chin, K., Sun, J., Chen, L., Hanover, J.A. & Rodgers, G.P. (2005) The hydroxyurea-induced small GTP-binding protein SAR modulates gamma-globin gene expression in human erythroid cells. Blood, 106, 3256–3263.

Taylor, J.G., Ackah, D., Cobb, C., Orr, N., Percy, M.J., Sachdev, V., Machado, R., Castro, O., Kato, G.J., Chanock, S.J. & Gladwin, M.T. (2008) Mutations and polymorphisms in hemoglobin genes and the risk of pulmonary hypertension and death in sickle cell disease. American Journal of Hematology, 83, 6–14.

Woolfe, A., Goodson, M., Goode, D.K., Snell, P., McEwen, G.K., Vavouri, T., Smith, S.F., North, P., Callaway, H., Kelly, K., Walter, K., Abnizova, I., Gilks, W., Edwards, Y.J., Cooke, J.E. & Elgar, G. (2005) Highly conserved non-coding sequences are associated with vertebrate development. PLoS Biology, 3, e7.

Zeng, Y.T., Huang, S.Z., Ren, Z.R., Lu, Z.H., Zeng, F.Y., Schechter, A.N. & Rodgers, G.P. (1995) Hydroxyurea therapy in beta-thalassemia intermedia: improvement in haematological parameters due to enhanced beta-globin synthesis. British Journal of Haematology, 90, 557–563.

Zimmerman, S.A., Schultz, W.H., Davis, J.S., Pickens, C.V., Mortier, N.A., Howard, T.A. & Ware, R.E. (2004) Sustained long-term hematologic efficacy of hydroxyurea at maximum tolerated dose in children with sickle cell disease. Blood, 103, 2039–2045.