A Twins Heritability Study on Alpha Hemoglobin Stabilizing Protein (AHSP) Expression Variability

Mei I. Lai,[1] Chad Garner,[3,4] Jie Jiang,[2] Nicholas Silver,[2] Steve Best,[2] Stephan Menzel,[2] and Swee L. Thein[2]

[1] *Department of Pathology, Universiti Putra Malaysia, Malaysia*

[2] *Molecular Haematology, Division of Gene and Cell Based Therapy, King's College London School of Medicine, United Kingdom*

[3] *Epidemiology Division, Department of Medicine, University of California, United States of America*

[4] *Department of Environmental Health, Science and Policy, University of California, United States of America*

Cytotoxic precipitation of free α-globin monomers and its production of reactive oxygen species cause red cell membrane damage that leads to anemia and eventually ineffective erythropoiesis in β-thalassemia. Alpha hemoglobin stabilizing protein (AHSP) was found to bind only to free α-globin monomers creating a stable and inert complex which remains soluble in the cytoplasm thus preventing harmful precipitations. Alpha hemoglobin stabilizing protein was shown to bind nascent α-globin monomers with transient strength before transferring α-globin to β-globin to form hemoglobin tetramer. A classical twin study would be beneficial to investigate the role of genetics and environment in the variation of alpha hemoglobin stabilizing protein expression as this knowledge will enable us to determine further investigations with regards to therapeutic interventions if alpha hemoglobin stabilizing protein is to be a therapeutic agent for β-thalassemia. This study investigates the heritability influence of alpha hemoglobin stabilizing protein expression and factors that may contribute to this. Results indicated that a major proportion of alpha hemoglobin stabilizing protein expression was influenced by genetic heritability (46%) with *cis*-acting factors accounting for 19% and *trans*-acting factors at 27%.

Keywords: twins study, AHSP, phenotypic variance

A screening of gene expressions controlled by GATA-1, an erythroid transcription factor, by Kihm et al. (2002) led to the discovery and study of alpha hemoglobin stabilizing protein (*AHSP*) gene as a molecular chaperone of α-globin chains (Kihm et al., 2002; Miele et al., 2001). AHSP was first identified by Miele et al. in 2001 as an erythroid differentiation-related factor (EDRF) that could be useful as a diagnostic marker for transmissible spongiform encephalopathy (TSE). As a chaperone, AHSP was found to bind only to α-globin chains and not to β-globin or Hb A (Kihm et al., 2002). AHSP binds to both reduced (ferrous) and oxidized (ferric) state of α-hemoglobin, preventing its harmful effects and the generation of reactive oxygen species (Feng et al., 2005). AHSP acts by binding to newly formed α-globin with an intermediate strength which is disrupted in the presence of sufficient β-hemoglobin. AHSP will then dislodge from α-globin and allow the formation of Hb A (Baudin-Creuza et al., 2004; Feng et al., 2004; Kihm et al., 2002; Mollan et al., 2010; Yu et al., 2007).

Studies have not only indicated the role of AHSP in α-globin protein folding for Hb A formation but also in modifying the severity of β-thalassemia. *AHSP* gene knock-out in mouse models has shown morphologies similar to β-thalassemia phenotype, whereby erythrocytes have inclusion bodies and decreased lifespan. These animals also have splenomegaly and bone marrow hyperplasia similar to β-thalassemic mice (Kihm et al., 2002; Kong et al., 2004). Previously we reported that a certain AHSP haplotype has a lower level of expression and was related to a more severe phenotype of β-thalassemia (Lai et al., 2006). The allelic frequency of this AHSP haplotype was also found to be higher in a group of Hb E/ β-thalassemia patients compared to their non-thalassemic control group in Thailand by Viprakasit et al., 2004. However, when they analyzed this patient group with a non-thalassemic control group from another province, the AHSP allele frequency and haplotypes were more similar (Viprakasit et al., 2004). Population analysis of the *AHSP* gene by dos Santos et al. (2008) suggested that the varied expression could be due to an Oct-1 binding site polymorphism related to the haplotype which alters and impairs AHSP promoter activity.

Received 15 July, 2010; accepted 24 September, 2010.

Address for correspondence: Mei I. Lai, Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Malaysia. E-mail: laimeii@medic.upm.edu.my

A structural mutation, AHSPN751 has shown to reduce AHSP's capacity to inhibit the reactive oxygen species production by α-hemoglobin (Brillet et al., 2010). Studies by Viprakasit et al. (2004) on the Thai population and Wang et al. (2010) in the Southern Chinese population have found no significant association between AHSP polymorphisms and β-thalassemia.

Studies have shown AHSP expression to be highly correlated with α-globin expression in both normal and β^0 thalassemia erythroblasts (dos Santos et al., 2004; Varrichio et al., 2009). Varricchio et al. (2009) reported that excess α-globin expression influences a feedback mechanism that regulate AHSP transcription, while dos Santos et al. (2008b) indicated that iron may play a role in activating the AHSP transcription process through iron regulatory proteins (IRPs). Variations in different studies may be due to different factors influencing the expression of *AHSP* gene. To assess the different factors that may modulate the AHSP expression, we carried out a classical twin heritability study in unselected twins. Results showed that 46% of AHSP expression was influenced by genetic factors while 27% and 28% were contributed by specific environmental factors and common environmental factors, respectively.

Design and Methods

Study Population

Subjects were 304 pairs of Caucasian, same-sex unselected adult monozygous (MZ) and dizygous (DZ) twins aged 20–83 years from St. Thomas' UK Adult Twin Registry (Spector & MacGregor, 2002). Informed consent was obtained prior to blood collection. This study was approved by King's College Hospital's local ethical committee (LREC 11-03-225).

Laboratory Studies

Confirmation of twins' zygosity through microsatellite typing analyses. Extraction of DNA was done using standard protocols. Dinucleotide microsatellite repeats were used for twin type determination as they are

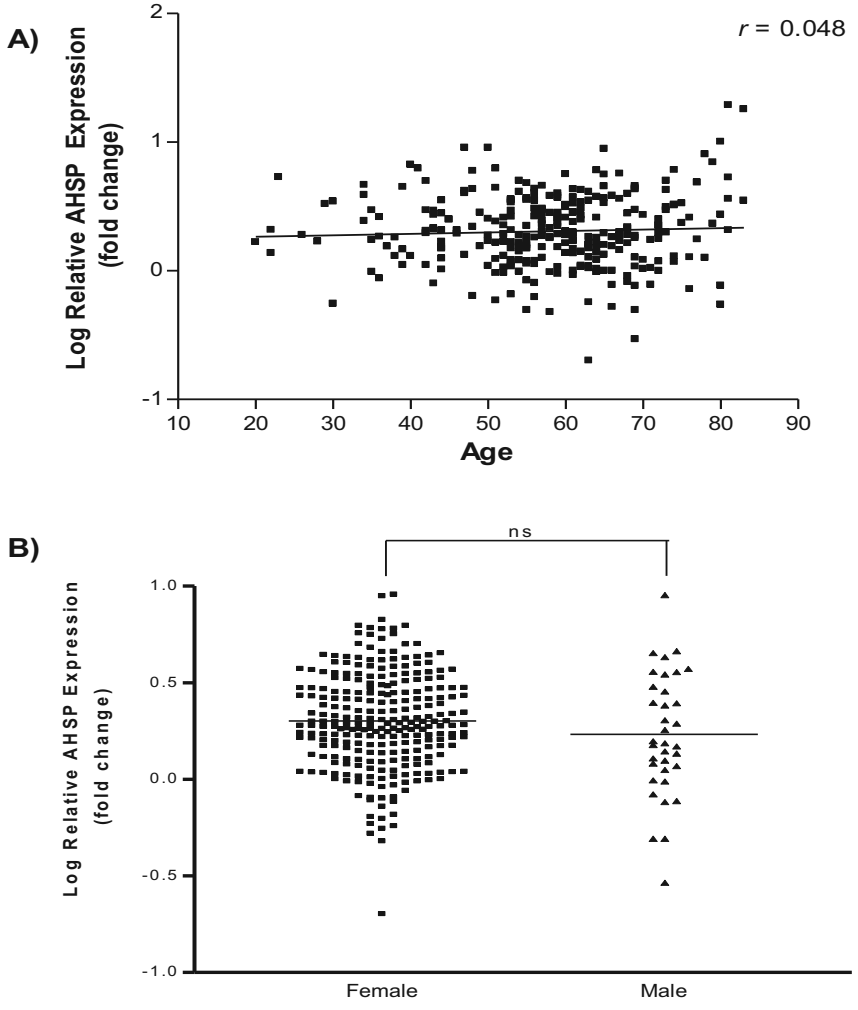

Figure 1

Influence of age and sex on the log relative AHSP expression of the twins. A) Relationship of age and log relative AHSP expression. B) Log relative AHSP expression between males and females. There were no significant influences of age and sex on the expression of AHSP (p = .4052 and p =.1417, respectively).

https://doi.org/10.1375/twin.13.6.567 Published online by Cambridge University Press

highly polymorphic. We used a panel of 8 microsatellite primer sets as the probability for a DZ twin to be misclassified as MZ is less than 1 in 1000. Primer sequences and PCR conditions are available upon request. PCR amplicons were genotyped using ABI Prism® 3100 Sequence Analyzer. Fragment sizing and allele calling were performed using Genotyper® 2.5 (Applied Biosystems, Warrington, UK).

Real-time quantitative reverse transcription-PCR. RNA from peripheral blood reticulocytes were extracted, isolated and transcribed as previously described (Lai et al., 2006). AHSP expression was assayed in each twin pair using TaqMan® hydrolysis probe assay (Applied Biosystems) and normalized to *GAPDH* (TaqMan® Gene Expression Assay; Applied Biosystems).

Statistical Analyses

Structural equation modeling with the Mx package was used for variance component analysis. This analysis calculates the maximum likelihood of a parameter to control the phenotypic variation of a trait. The details of the analysis have been described previously (Lai et al., 2006, Neale & Cardon, 1992). In our study, 3 models; ACE, ADE and AE were tested using the Mx package (A: additive genetic effect; D: dominance genetic effect; C: common environmental effect; E: specific environmental effect). The ACE model indicates the influence of additive genetic and common environmental effects on the *AHSP* phenotype, ADE model is the influence of additive and dominance genetic effects and AE model represents only the additive genetic effects. A χ^2 goodness-of-fit analysis was done to test the model most suitable for our data with the smallest value indicating the best fit.

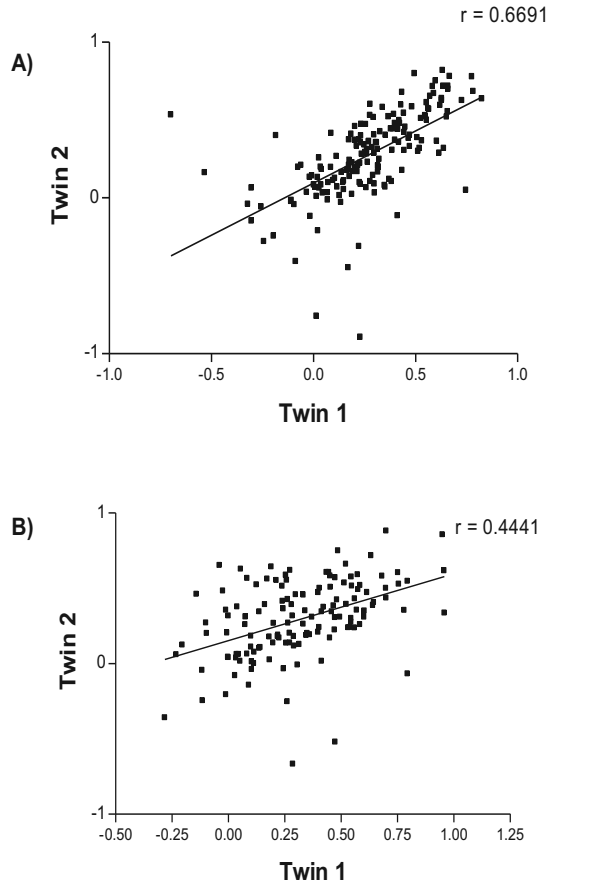

Figure 2

Correlation of log relative AHSP expression between twin 1 and twin 2 in (A) MZ twin pairs and (B) DZ twin pairs. Coefficient correlation (r), rMZ = 0.669 ($p < .0001$) and rDZ = 0.444 ($p < .0001$). The higher correlation between monozygotic twins compared to dizygotic twins implies the influence of genetic components on the variations observed in AHSP expression.

Results

A total of 304 unselected Caucasian, same-sex, healthy twin pairs aged 20-83 years with the average age of 57 years (min. = 20, max. = 83, *SD* = 11.6) were studied. Only 295 twin pairs were analyzed as 9 pairs were excluded due to microsatellite typing failures in one or both twins. Out of the 295 twin pairs, 133 pairs were classified as dizygotic (DZ) (male = 13 pairs, female = 120 pairs) and 162 were monozygotic (MZ) (male = 23 pairs, female = 139 pairs). Twins pairs with alleles differed by at least 1 locus was immediately classified as DZ twins. However, 9 twin pairs were designated as uncertain MZ because their probability of misclassification was larger than 10^{-3}.

Distribution of AHSP expression was positively skewed with a mean of 2.32 ± 1.38 fold-change and had to be log-transformed to fit the normal distribution for further statistical analyses. The fold-change was calculated using the $\Delta\Delta C_t$ comparative method relative to a standard calibrator with GAPDH as the housekeeping control. Each analysis was done in duplicates and normalized to GAPDH.

Influences of common non-genetic parameters, i.e. age and sex of the twins, on the expression of AHSP were determined by randomly selecting one twin from each pair for analysis. Correlation coefficient (*r*) and simple linear regression analysis were used to analyze the influence of age and a student *t* test was used to analyze the influence of sex. Analyses showed there were no significant correlation between age ($r = .05$ and $p = .405$) and sex ($p = .142$) with AHSP expression, respectively (Figure 1).

A Pearson correlation coefficient analysis (*r*) was carried out to determine intra-class correlation between the MZ and DZ twins. A higher correlation coefficient (*r*) indicated a significant degree of genetic influence on AHSP expression. Pearson correlation analysis demonstrated that the log relative AHSP expression values were more similar in MZ twins compared to DZ twins. The intra-class correlation coefficient (*r*) in MZ twins was 0.669 ($p < .0001$) and *r*DZ = 0.444 ($p < .0001$) indicating that genetic components do contribute a certain degree to the variations of AHSP expression level (Figure 2). From the Pearson correlation coefficient analysis, we decided to investigate the variance

https://doi.org/10.1375/twin.13.6.567 Published online by Cambridge University Press

Table 1

Results of Variance Components Analysis Before and After Haplotype Adjustments

Model	d.f.	χ^2	h^2	c^2	d^2	e^2
No haplotype adjustment						
ACE	3	0.000	0.46	0.28	—	0.27
ADE	3	3.948	0.73	—	0.00	0.26
AE	4	3.948	0.73	—	—	0.26
Adjusted for haplotype						
ACE	3	0.000	0.27	0.33	—	0.40
ADE	3	5.012	0.62	—	0.00	0.37
AE	4	5.012	0.62	—	—	0.37

Note: h = heritability effect; c = common environment effect; d = dominance genetic effect; e = specific environment effect. The three models, additive genetic and common environmental effects (ACE), additive and dominance genetic effects (ADE) and additive genetic effects only (AE) were analyzed using $\chi 2$ goodness-of-fit test to calculate the most likely model to control the phenotypic variation of AHSP, whereby a smaller χ 2 value indicates a better fit. The model ACE without AHSP haplotype adjustment indicated 46% of the variance was due to genetic factors. When adjusted for the haplotype, only 27% of the variance was due to genetic factors.

components influencing AHSP expression particularly the genetic component.

Through structural equation modeling statistical analyses, we found that the ACE model is the best-fit model to represent the variations controlling the AHSP expression. The heritability (h^2) estimate for additive genetic effect under this model was 0.46 (46%) with common environmental effect (c^2) contributing 28% (0.28) and 27% (0.27) due to specific environmental effect (e^2). The results are shown in Table 1. We reported that the major haplotype (Clade A) of the *AHSP* gene has a higher expression level in non-thalassemic individuals compared to those with Clade B (Lai et al., 2006). An analysis of variance that included one twin from each twin pair showed that the haplotypes accounted for approximately 5% of the variation in the logAHSP trait values.

Discussion

A wide range of phenotypic variance in β-thalassemia even though with similar β genotype has led researchers to look at *trans*-acting modifying factors involved in the pathophysiology of β-thalassemia. *Trans*-acting factors that have been extensively studied gave us more clues in piecing together the factors involved in β-thalassemia phenotypic variability. The discovery of *AHSP* as a molecular chaperone for α-globin chains not only facilitated our understanding in this subject but also its role in hemoglobin synthesis (Kong et al., 2004; Lai et al., 2006; Mollan et al., 2010; Yu et al., 2007).

A classical twin study allows us to study the extent of genetic influences on AHSP expression. The magnitude of increased MZ similarity is a measure for the relative importance of genetically-caused variability in comparison with environmentally-caused variability. Although occasionally DZ pairs can share all genotypes by chance, the likelihood of this occurring decreases when more genotypes are compared and when the actual alleles present in the respective twin pairs are informative. The probability for misclassification in our study was less than one in a thousand for the majority of the twin pairs (94%). We have shown that there are higher AHSP expression similarities between the monozygotic twin sets compared to the dizygotic twin sets ($p < .0001$) indicating a definite influence of genetic components in the variation of AHSP expression.

Our study has indicated the different variance components that affect AHSP expression. We can conclude here that genetic influence plays the major role in determining the variable expression of AHSP (46%) followed by common environmental effects and specific environmental effects at 28% and 27%, respectively. The haplotype differences of the *AHSP* gene accounts for a subtle variation in the AHSP expression which indicates other genetic factors that may play a bigger role than AHSP haplotypes in determining the expression of AHSP. Other factors that may play a role are discussed below.

A study by Gallagher et al. demonstrated that GATA-1 and Oct-1 transcription factors were needed for high expression of AHSP in vitro. GATA-1 and Oct-1 binds to *AHSP* gene promoter region at -98 to -104 and +238 to +250 binding sites, respectively. AHSP promoter activity was shown to reduce to background levels in luciferase assays when mutations were introduced into those binding sites (dos Santos et al., 2008; Gallagher et al., 2005).

dos Santos et al. found an iron-responsive element-like stem-loop structure at the poly-adenylation site of the AHSP mRNA that facilitates in the regulation of AHSP mRNA stability. Using K562 cells, their study showed increased AHSP gene transcription when iron was present, but it also destabilizes mature AHSP mRNA at the same time. A mutation in the IRE-like structure causes the AHSP mRNA to be more susceptible to endonucleolytic cleavage and thus more prone to denaturation (dos Santos et al., 2008b). This group in the same year reported a mutation, AHSPN75I, in which the AHSP was expressed at the normal levels but

https://doi.org/10.1375/twin.13.6.567 Published online by Cambridge University Press

was less effective in reducing the effects of oxidative stress from α-hemoglobin (dos Santos et al., 2008).

No other *cis*-acting polymorphisms were found to be correlated to the expression of AHSP although few mutations were reported (dos Santos et al., 2008; Viprakasit et al., 2004; Wang et al., 2010). Expression of AHSP has been highly correlated with α-globin expression (dos Santos et al., 2004; Varrichio et al., 2009). AHSP expression was observed to be independent of both γ- and β-globin expression but was positively-correlated to the levels of excess α-globin [α – (γ + β)] in normal and β^0-thalassemia erythroblasts (Varrichio et al., 2009) substantiating its role as an α-globin chaperone.

These studies have shown that there are multiple genetic influences that cause the variability in AHSP expression. Some of them could be a form of regulatory feedback mechanism that the cell uses to balance the transcription of AHSP that would explain the role and function of AHSP further. Understanding these factors and the role that they play will allow us to explore the possibility of AHSP being a therapeutic agent to modify the phenotype of β-thalassemia further.

Funding

This work was supported by an MRC grant (G0000111, ID51640) to S. L. Thein.

Acknowledgment

We would like to thank Tim Spector for providing us with the samples for this study.

Authorship and Disclosures

MIL was the principal investigator and takes primary responsibility for the paper. MIL and NS performed the laboratory work for this study. CG and MIL performed the statistical analyses, JJ, SB, SM and SLT coordinated the research. The authors reported no potential conflicts of interest.

References

Baudin-Creuza, V., Vasseur-Godbillon, C., Pato, C., Prehu, C., Wajcman, H., & Marden, M. C. (2004). Transfer of human α- to β-hemoglobin via its chaperone protein: Evidence for a new state. *Journal of Biological Chemistry*, *279,* 36530–36533.

Brillet, T., Baudin-Creuza, V., Vasseur, C., Domingues-Hamdi, E., Kiger, L., Wajcman, H., Pissard, S., & Marden, M. C. (2010). Alpha-hemoglobin stabilizing protein (AHSP), a kinetic scheme of the action of a human mutant, AHSPV56G. *Journal of Biological Chemistry*, *285,* 17986–17992.

dos Santos, C. O., Duarte, A. S. S., Saad, S. T. O., & Costa, F. F. (2004). Expression of -hemoglobin stabilizing protein gene during human erythropoiesis. *Experimental Hematology*, *32*, 157–162.

dos Santos, C. O., Zhou, S., Secolin, R., Wang, X., Cunha, A. F., Higgs, D. R., Kwiatkowski, J. L., Thein, S. L., Gallagher, P. G., Costa, F. F., & Weiss, M. J. (2008). Population analysis of the alpha hemoglobin stabilizing protein (AHSP) gene identifies sequence variants that alter expression and function. *American Journal of Hematology*, *83,* 103–108.

dos Santos, C. O., Dore, L. C., Valentine, E., Shelat, S. G., Hardison, R. C., Ghosh, M., Wang, W., Eisenstein, R. S., Costa, F. F., & Weiss, M. J. (2008b). An iron responsive element-like stem-loop regulates alpha hemoglobin stabilizing protein mRNA. *Journal of Biological Chemistry*, 283, 26956–26964.

Feng, L., Gell, D. A., Zhou, S., Gu, L., Kong, Y., Li, J., Hu, M., Yan, N., Lee, C., Rich, A. M., Armstrong, R. S., Lay, P. A., Gow, A. J., Weiss, M. J., Mackay, J. P., & Shi, Y. (2004). Molecular mechanism of AHSP-mediated stabilization of α-hemoglobin. *Cell*, *119*, 629–640.

Feng, L., Zhou, S., Gu, L., Gell, D.A., Mackay, J. P., Weiss, M. J., Gow, A. J., & Shi, Y. (2005). Structure of oxidized α-haemoglobin bound to AHSP reveals a protective mechanism for haem. *Nature, 435,* 697–701.

Gallagher, P. G., Liem, R. I., Wong, E., Weiss, M. J., & Bodine, D. M. (2005). GATA-1 and Oct-1 are required for expression of the human α-hemoglobin-stabilizing protein gene. *Journal Biological Chemistry*, *280,* 39016–39023.

Kihm, A. J., Kong, Y., Hong, W., Russell, J. E., Rouda, S., Adachi, K., Simon, M. C., Blobel, G. A., & Weiss, M. J. (2002). An abundant erythroid protein that stabilizes free α-haemoglobin. *Nature, 417,* 758–763.

Kong, Y., Zhou, S., Kihm, A. J., Katein, A. M., Yu, X., Gell, D. A., Mackey, J. P., Adachi, K., Foster-Brown, L., Louden, C. S., Gow, A. J., & Weiss, M. J. (2004). Loss of α-hemoglobin–stabilizing protein impairs erythropoiesis and exacerbates β-thalassemia. *The Journal of Clinical Investigation, 114,* 1457–1466.

Lai, M. I., Jiang, J., Silver, N., Best, S., Menzel, S., Mijovic, A., Colella, S., Jiannis, R., Garner, C., Weiss, M. J., & Thein, S. L. (2006). Alpha-haemoglobin stabilising protein is a quantitative trait gene that modifies the phenotype of beta-thalassaemia. *British Journal of Haematology, 133,* 675–682.

Miele, G., Manson, J., & Clinton, M. (2001). A novel erythroid-specific marker of transmissible spongiform encephalopathies. *Nature Medicine*, *7*, 361–364.

Mollan, T. L., Yu, X., Weiss, M. J., & Olson, J. S. (2010). The role of alpha-hemoglobin stabilizing protein in redox chemistry, denaturation, and hemoglobin assembly. *Antioxidants & Redox Signaling*, *12*, 219–231.

Neale, M. C., & Cardon, L. R. (1992). *Methodology for genetic studies of twins and families* (NATO ASI Series D: Behavioural and Social Sciences, Vol. 67).

Spector, T. D., & MacGregor, A. J. (2002). The St. Thomas' UK adult twin registry. *Twins Research, 5,* 440–443.

Varricchio, L., Fabucci, M. E., Alfani, E., Godbold, J., & Migliaccio, A. R. (2009). Compensated variability in

https://doi.org/10.1375/twin.13.6.567 Published online by Cambridge University Press

the expression of globin-related genes in erythroblasts generated ex vivo from different donors. *Transfusion*, *50*, 672–684.

Viprakasit, V., Tanphaichitr, V. S., Chinchang, W., Sangkla, P., Weiss, M. J., & Higgs, D. R. (2004). Evaluation of alpha hemoglobin stabilizing protein (AHSP) as a genetic modifier in patients with β thalassemia. *Blood*, *103*, 3296–3299.

Wang, Z., Yu, W., Li, Y., Shang, X., Zhang, X., Xiong, F., & Xu, X. (2010). Analysis of alpha-hemoglobin-stabilizing protein (AHSP) gene as a genetic modifier to the phenotype of b-thalassemia in Southern China. *Blood Cells, Molecules, and Diseases*, 45, 128–132.

Yu, X., Kong, Y., Dore, L. C., Abdulmalik, O., Katein, A. M., Zhou, S., Choi, J. K., Gell, D., Mackay, J. P., Gow, A. J., & Weiss, M. J. (2007). An erythroid chaperone that facilitates folding of α-globin subunits for hemoglobin synthesis. *Journal of Clinical Investigation*, *117*, 1856–1865.

https://doi.org/10.1375/twin.13.6.567 Published online by Cambridge University Press