

A candidate gene study of F cell levels in sibling pairs using a joint linkage and association analysis

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Abstract

Introduction Fetal haemoglobin (Hb F) and fetal cell (FC) levels in adults show considerable variation and the heritability of FC levels has been estimated to be 89% in the healthy European population; measured genotype analyses have shown the trait to be influenced by several genetic factors. In addition to the β -globin gene complex on chromosome 11p, linkage analyses have reported loci affecting FC levels on chromosomes Xp22.2–p22.3 and 6q23.

Methods We have genotyped a sample of approximately 300 unselected, same sex, dizygotic twin pairs from the St. Thomas' UK Adult Twin Register for markers in these three regions and carried out a linkage analysis of FC levels. We also used a new method to simultaneously test for linkage and allelic association, and association caused by population stratification or admixture.

Results There was no evidence for linkage of FC levels to chromosomes Xp22.2–p22.3 and 6q23. However, the β -globin cluster was shown to be significantly linked and to be responsible for approximately one-third of the additive genetic variance in FC levels in the sample.

Conclusions The report represents the first application of this method to a sample of nonsimulated data and demonstrates the effectiveness of the approach. Combined linkage and association analysis of the β -globin complex showed a strong association between the *XmnI*- $\zeta\gamma$ site and FC levels and that the observed association is not an artifact of recent population admixture or stratification.

Keywords F cells, sib-pairs, linkage analysis, association analysis.

Introduction

In normal adults, the synthesis of fetal Hb (Hb F) is reduced to very low levels, with the vast majority having trace amounts of Hb F¹. The Hb F is unevenly distributed and restricted to a subset of erythrocytes named F cells (FC)². Synthesis of much higher levels of Hb F, however, can persist into adults as part of a heterogeneous group of conditions referred to as hereditary persistence of fetal haemoglobin (HPFH)¹. Rare

forms of HPFH are caused by large deletions within the β globin gene cluster on chromosome 11p or by point mutations in the promoters of the γ -globin genes. The levels of Hb F in heterozygotes for these forms of HPFH are in the range of 5–30% of total haemoglobin and the Hb F is evenly distributed among the F cells in a 'pancellular' fashion. However, there is a more common type of HPFH characterized by a modest increase of Hb F (up to 4% of total haemoglobin) that is distributed in an uneven (heterocellular) fashion among the F cells. Surveys of FC distribution in normal individuals suggest that heterocellular HPFH may be present in ~10% of the population^{3–6}, and represents the elevated levels of Hb F and FC at the upper tail of the trait distribution. Unlike the rare forms of HPFH which demonstrate a clear Mendelian inheritance as alleles of the β -globin locus, no mutations have been identified within the β -globin complex in heterocellular

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HPFH. Furthermore, in many families the high FC phenotype segregates independently of the β -complex, implicating the presence of *trans*-acting factors^{7,8-11}.

Recently, two such loci involved in the control of FC production have been mapped by linkage analysis. One locus has been mapped on chromosome 6q23 in an extensive, inbred Asian Indian kindred with β thalassaemia¹²⁻¹³. The other locus (FC production or FCP locus) which is associated with variation in FC levels in sickle cell disease has been mapped to the Xp22.2-p22.3 region¹⁴. Apart from the two *trans*-acting loci, other factors known to influence Hb F/FC levels in adults include age (levels fall as one becomes older)¹⁵, sex (women have higher levels than men)⁴, sequence variants within the β -globin complex (including a C-T polymorphism at position -158 of the γ globin gene¹⁶ and a four base pair (AGCA) deletion at positions -222 to -225 of the γ globin gene¹⁷), and the inheritance of β -thalassaemia¹⁸.

The linkage of chromosomes 6q23, Xp22.2-p22.3 and the β -globin complex to the maintenance of high FC levels indicates that there are genetic factors influencing the expression of the high FC trait. A recent analysis of monozygotic and dizygotic twin pairs from the St. Thomas' UK Adult Twin Registry estimated the additive genetic variance components to be 89% of the total phenotypic variance in FC levels in the healthy, adult European population¹⁹. It is not known if the *trans*- and *cis*-acting factors that have been implicated in the aetiology of HPFH have an effect on FC levels in the general population or the extent to which they account for the observed heritability. To address this issue, we have carried out a candidate gene linkage study of FC levels at markers on chromosomes 6q23, Xp22.2-p22.3 and the β -globin complex using a sample of dizygotic (DZ) twin pairs. There was no evidence for linkage of FC levels to chromosomes 6q23 or Xp22.2-p22.3; however, there was evidence of linkage to the β -globin complex on chromosome 11p. A joint analysis of linkage between FC levels and the β -globin complex, and allelic association between the putative quantitative trait loci (QTL) and the *XmnI*- γ and γ -4 bp deletion sites was carried out using a new method developed by Fulker *et al.*²⁰ and extended by Cardon²¹. Significant evidence for linkage of FC levels to the β -globin complex and for allelic association between the *XmnI*- γ polymorphism and FC levels are shown.

Materials and methods

Family material and F cell assays

The population sample in the study consisted of 316 same sex, DZ twin pairs of European descent from the St. Thomas' UK Adult Twin Registry²². The sample consisted of 288 female twin pairs and 28 male twin pairs ranging in age from 20 to 73 years. The Registry is essentially volunteer based, with individuals unselected for trait or disease. Zygosity was determined by a standard questionnaire and confirmed by DNA analysis of highly polymorphic short tandem repeats (STRs).

F cell assays were performed on peripheral blood collected in EDTA (as anticoagulant) using a monoclonal mouse anti- γ globin chain antibody by fluorescence-activated cell sorting (FACS) and 10⁴ cells were counted per assay in all cases²³.

Genotyping and marker data

DNA was extracted from peripheral blood leucocytes using standard procedures. The *XmnI*- γ site at position -158 of the γ -globin gene and the γ -4 bp deletion at positions -224 to -221 relative to the mRNA cap site of the γ globin gene were detected by restriction enzyme analysis (*XmnI* and *Fnu4HI*, respectively) of specifically amplified PCR products²⁴.

Genotyping for the marker D6S976 (*AFM095wfl1*) was performed by PCR amplification of the microsatellite and analysed on denaturing 7 M urea 6% polyacrylamide gels as described²⁵. The microsatellites were detected using a ³²P-labelled (CA)_n probe. Genotyping for the other markers — D6S270, a (CA)_n repeat in the BAC clone 521H20 (b521H20 CA), the β -globin gene cluster [$\delta\beta$ (CA)_n], D6S1626, D6S292, DXS1060, DXS1223, DXS8051 and DXS7108 — was performed by carrying out PCR with fluorescently labelled oligonucleotides and detecting the products on an ABI377 sequencer (Perkin Elmer). Gels were analysed using Genescan analysis (Perkin Elmer) and the GENOTYPER program²⁵. The marker b521H20CA was a newly isolated (CA)_n microsatellite whereas the other microsatellite marker loci were selected from the Généthon database²⁶.

Figure 1 is a representation of the markers on the β -globin gene cluster, chromosome 6q23 and chromosome Xp22.2-p22.3 used in the analysis. The between-marker distances are shown in centimorgans (cM) or kilobases (kb). All of the markers were microsatellite CA repeat polymorphisms with the exception of the *XmnI*- γ polymorphism and the γ -4 bp deletion. Map distances and the locations of the β -globin complex markers were assigned based on published data^{13,26} (Genbank HUMHBB), and the multipoint IBD information provided by the map of markers was calculated using MAPMAKER/SIBS. Over 80% of the IBD information is extracted with the markers in chromosomes 6q23 and Xp22.2-p22.3, and the marker $\delta\beta$ (CA)_n in the β -globin complex extracted approximately 44% of the IBD information. Maximum likelihood estimates of the allele frequencies at each marker were calculated using the program SPLINK²⁷⁻²⁸.

Using an unpublished method for identifying potential genotype errors in sib pairs without parental data, we eliminated some of the genotype data from the analysis. Some errors could be resolved by considering individual genotypes; however, 17 sib pairs were eliminated from the chromosome 6 analysis, 8 were eliminated from the chromosome X analysis and 7 from the chromosome 11 analysis, because multiple potential genotype errors were observed in the data. In total, there were 298, 308 and 309 sib pairs used in the analysis of chromosome 6, X and the β -globin complex, respectively.

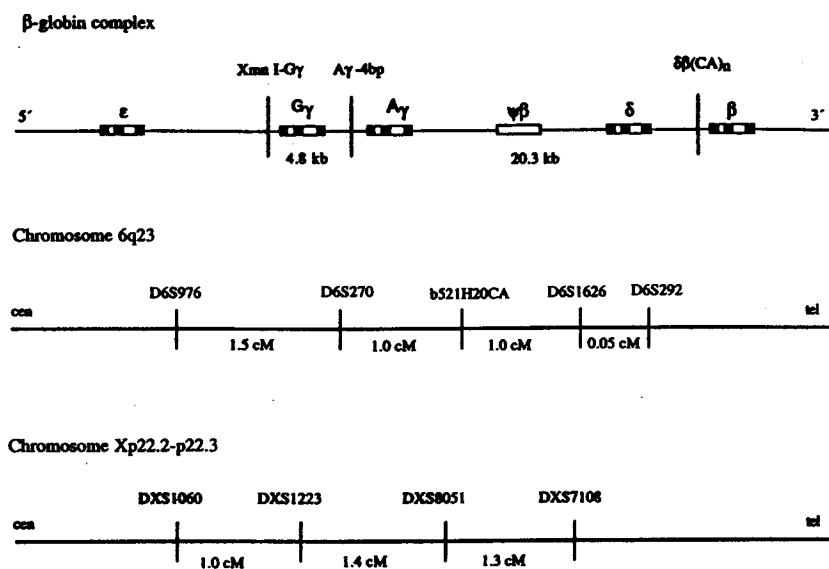


Figure 1 Maps of the markers used in the analyses. Distances between markers on chromosomes X and 6 are shown in centimorgans (cM).

Linkage analysis

Variance components linkage analysis was carried out using the MULTIC program²⁹ with multipoint IBD estimates for chromosomes 6q23 and Xp22.2-p22.3, and single point estimates for marker $\delta\beta(CA)_n$ obtained from the MAPMAKER/SIBS program³⁰.

A likelihood ratio test was used to test if the major genetic variance component was significantly different from 0, where the difference in the $-2 \log_e$ likelihood between the models is assumed to asymptotically follow a 50 : 50 mixture of a chi-squared distribution with 1 d.f. and a point mass at 0³¹.

Combined linkage and association analysis

Fulker *et al.*²⁰ have recently described a novel likelihood-based method for joint analysis of linkage and allelic association between a diallelic marker and a QTL using the classical biometrical model³². The method, which tests for true allelic association vs. association caused by population admixture, is designed for raw sibship data and does not require parental data. The test for spurious association caused by population admixture is based on the assumption that population stratification will influence sib pair means but not sib pair differences.

Cardon²¹ has extended the Fulker *et al.*²⁰ method to a multiple regression framework. Denoting X_{1i} and X_{2i} as the trait values of the arbitrarily ordered first and second sibs from the *i*th sibship, I_{mi} as the IBD information at the marker locus *M*, and π_{mi} as the mean alleles shared IBD, Carey and Williamson³³ and Fulker *et al.*³⁴ have shown that the regression equation

$$E(X_{2i} | I_{mi}) = \alpha + b_1 X_{1i} + b_2 \pi_{mi} + b_3 X_{1i} \pi_{mi}$$

is a direct test of linkage between the marker and QTL in b_3 and b_1 is a test of the shared, or common, environmental effect. Cardon has extended this model to test for allelic association, under the assumption of Fulker *et al.*²⁰. Cardon describes two multiple regression models. The first model is a test of linkage and general association in which the association is measured in a single parameter reflecting the expected additive value of the co-sibling. A single contrast-coded variable, A_2 is included in the regression model

$$E(X_{2i} | I_{mi}) = \alpha + b_1 X_{1i} + b_2 \pi_{mi} + b_3 X_{1i} \pi_{mi} + b_4 A_2$$

with contrast-codes constructed to reflect the expected additive genetic value for sibling 2 in each of the nine possible sibling pair genotype combinations. The second model is a within-pairs test that models sibling pair differences. In the within-pairs test, the contrast-coded variable A_2 is replaced by a similar contrast-coded variable W_2 , which represents the component of the overall additive genetic variance for sibling 2 and reflects the expected sibling differences attributed to the QTL, so that the regression model becomes

$$E(X_{2i} | I_{mi}) = \alpha + b_1 X_{1i} + b_2 \pi_{mi} + b_3 X_{1i} \pi_{mi} + b_4 W_2.$$

Thus b_4 in the above equation will not be affected by any sampling-specific effects.

Joint linkage and association analysis based on the above methods was carried out using the Statistical Analysis System (SAS)³⁵. The nine possible sibling genotype configurations were assigned based upon the genotypes at the diallelic loci *XmnI*- γ and $A\gamma$ -4 bp. We have created contrast-codes under an allele-dose model rather than the additive genotype effect model described in Fulker *et al.*²⁰ and Cardon²¹. Table 1 shows the contrast coding for each sibling genotype

Table 1 Contrast coding for allele-dose model of additive genetic effect

Genotype		Allele 1 doses		A_1	W_1
Sib 1	Sib 2	Sib 1	Sib 2		
A_1A_1	A_1A_1	2	2	2	0
A_1A_1	A_1A_2	2	1	1.5	-0.5
A_1A_1	A_2A_2	2	0	1	1
A_1A_2	A_1A_1	1	2	1.5	-0.5
A_1A_2	A_1A_2	1	1	1	0
A_1A_2	A_2A_2	1	0	0.5	0.5
A_2A_2	A_1A_1	0	2	1	-1
A_2A_2	A_1A_2	0	1	0.5	-1
A_2A_2	A_2A_2	0	0	0	0

configuration and the corresponding values of A_1 and W_1 , the overall mean and the within-pair mean, respectively. The vector of mean IBD sharing probabilities, π , was calculated from the distribution of single-point IBD probabilities at the marker $\delta\beta(CA)_n$ computed with the MAPMAKER/SIBS program.

The following multiple regression models were tested in the analysis:

$$1 \quad \chi_2 = b_1X_1 + b_2\pi + b_3X_1\pi$$

$$2 \quad \chi_2 = b_1X_1 + b_2\pi + b_3X_1\pi + b_4a$$

$$3 \quad \chi_2 = b_1X_1 + b_2\pi + b_3X_1\pi + b_4a_w$$

$$4 \quad \chi_2 = b_1X_1 + b_4a$$

Model 1 is equivalent to the linkage model described in Carey and Williamson³³ and Fulker *et al.*³⁴. Model 2 is an overall test of association with model 3 being the within-pairs test described above and in Cardon²¹. In the models, the regression coefficient associated with X_1 is an estimate of the sibling common environment, C^2 ; in model 4 it is $2C^2$. Model 4 is a test of association alone. The significance of the independent variables was measured with a *t*-test, with H_0 being all regression parameters equal to 0. The probability of *t* for the regression parameters and the total R^2 for the regression are reported for each model.

Results

Linkage analysis

There was no evidence for the presence of major genetic factors on chromosomes 6q23 or Xp22.2-p22.3, with both regions showing maximum likelihood estimates of zero genetic effects. There was evidence for a major genetic effect at the β -globin cluster accounting for 37% of the total additive genetic variance (LRT = 7.0, $P = 0.004$) or 35% of the total phenotypic variance.

Table 2 Results of combined linkage and allelic association analysis for the $XmnI$ - $\text{C}\gamma$ and $A\gamma$ -4 bp deletion sites. Descriptions of models 1-4 are given in the Methods. The regression coefficients for the model parameters are shown over their corresponding significance levels

Model	Regression coefficients associated with the independent variables (β/P -value)				R^2
	X_1	$X_1\pi$	A_1	W_1	
$XmnI$-$\text{C}\gamma$					
1	0.29	0.37	—	—	0.25
	0.004	0.03			
2	0.27	0.31	-0.46	—	0.33
	0.004	0.06	< 0.001		
3	0.38	0.27	—	0.74	0.31
	< 0.001	0.10		< 0.001	
4	0.43	—	-0.47	—	0.32
	< 0.001		< 0.001		
$A\gamma$-4 bp					
1	0.29	0.37	—	—	0.25
	0.004	0.03			
2	0.30	0.33	-0.19	—	0.25
	0.004	0.05	0.03		
3	0.29	0.36	—	0.15	0.24
	0.005	0.03		0.41	
4	0.46	—	-0.20	—	0.24
	< 0.001		0.018		

Linkage and association analysis of β -globin gene cluster

Linkage disequilibrium between $XmnI$ - $\text{C}\gamma$ and $A\gamma$ -4 bp deletion sites was tested using the program ASSOCIATE³⁶. The program estimates a single disequilibrium parameter, D , and tests its significance by a single degree-of-freedom (d.f.), likelihood ratio χ^2 test. The $XmnI$ - $\text{C}\gamma$ and $A\gamma$ -4 bp deletion sites showed significant linkage disequilibrium ($\chi^2 = 72.0$, 1 d.f., $P < 0.0001$).

The results of the joint linkage and allelic association analysis are shown in Table 2. In the model, allele 1 represents the presence of the $XmnI$ - $\text{C}\gamma$ restriction site and the absence of the $A\gamma$ -4 bp deletion. Model 1 is a pure test of linkage and does not include the $XmnI$ - $\text{C}\gamma$ and $A\gamma$ -4 bp deletion genotypes explicitly in the model. The region shows evidence for linkage as would be expected from the results of the variance components analysis. Model 2 shows a significant overall association between the $XmnI$ - $\text{C}\gamma$ site and the FC QTL, with the regression parameter for association significant at $P < 0.001$. The $A\gamma$ -4 bp deletion site showed a less significant overall association effect, $P = 0.03$. The within-pairs test for pure allelic association was significant for the $XmnI$ - $\text{C}\gamma$ site ($P < 0.001$). The evidence for association shown for the $A\gamma$ -4 bp deletion site in model 2 was not shown in model 3, indicating that there is no statistical evidence for pure allelic association between the $A\gamma$ -4 bp deletion site and the FC QTL. Model 4 shows that 32% of the total variance in FC is accounted for with a model which includes only the common environmental effect and the effect from the allelic association with the $XmnI$ - $\text{C}\gamma$ site. This indicates that the observed linkage to the β -globin gene cluster

can be completely accounted for by the allelic association at the *XmnI*- $\text{C}\gamma$ site. The estimate of the common environmental effect varied depending upon the model but averaged 0.30 across all models with a standard deviation of 0.03. In a previous report Garner *et al.*¹⁹ estimated that the *XmnI*- $\text{C}\gamma$ site accounted for 13% of the total phenotypic variance of FC levels, using a linear regression approach which did not consider the family relationships or IBD at the β -globin complex. The variance components and multiple regression approaches described in this report gave relatively consistent and somewhat higher estimates of the effect and exemplify the differences that can be observed in the effect sizes using different methods. The $\Delta\gamma$ -4 bp deletion site is approximately 4.8 kb from the *XmnI*- $\text{C}\gamma$ site and the markers are in strong linkage disequilibrium with each other; however, the $\Delta\gamma$ -4 bp deletion site showed no evidence for allelic association to a FC QTL in the β -globin complex.

Discussion

The results presented show that loci on chromosomes 6q23 and Xp22.2-p22.3 do not have a significant effect on FC variation in this sample of healthy, adult European sibling pairs. Linkage to the chromosome Xp22.2-p22.3 locus was detected in a sample of predominantly Afro-Caribbeans where high FC levels are associated with sickle cell disease and the chromosome 6q23 locus was detected in a six-generation family from the Gujarat region of India with heterocellular HPFH and β -thalassaemia. The loci that have been identified in these two populations are associated with the inheritance of an extreme high FC trait, in the presence of other haematological traits. Their effect on the phenotypic variance observed in the general European population has been addressed by this study. It is conceivable that the findings on chromosomes 6q23 and Xp22.2-p22.3 represent linkages to Mendelian forms of heterocellular HPFH which are not represented in the sample used in the analyses presented here. Considering the results of Garner *et al.*¹⁹, who estimated the heritability of FC levels to be 89% in the general European population, and the results of the variance components analysis presented here, there are likely to be *trans*-acting loci affecting FC variance in the European population. This *trans*-acting effect could be caused by one or a few major genes, or it could be polygenic. In order to further explore both the chromosome 6q23 and Xp22.2-p22.3 loci, candidate gene studies will have to be carried out using families that are more phenotypically and demographically similar to the families in which the linkages were reported.

The variance components linkage analysis of the β -globin complex indicates that there is a major genetic effect at this locus in the European population which is accounting for as much as 42% of the observed additive genetic variance. Fifty-three percent of the additive genetic variance in FC must be due to unidentified *trans*-acting factors. A combined linkage and association analysis of the European sib pair

sample was carried out which showed that the linkage effect from the β -globin complex on FC levels can almost entirely be accounted for by the association with the *XmnI*- $\text{C}\gamma$ site. The combined effect of common sibling environment and the allelic association with the *XmnI*- $\text{C}\gamma$ site was shown to account for 32% of the total phenotypic variance in FC levels in this European sample. These results indicate that the *XmnI*- $\text{C}\gamma$ site is in very strong linkage disequilibrium with the high FC variant or may be the high FC QTL itself at the β -globin complex. The $\Delta\gamma$ -4 bp deletion site was shown not to be highly associated with high FC levels and its association effect does not exceed the linkage effect observed at the complex; the test of linkage and association account for 24% and 25% of the phenotypic variance in FC levels, respectively.

Using three different methods, the β -globin complex has been shown to have an effect on FC levels in the European sample. Garner *et al.*¹⁹ carried out a simple regression analysis of unrelated individuals' FC values with the *XmnI*- $\text{C}\gamma$ site as an independent variable, and estimated that the genotype accounted for 13% of the total phenotypic variance in FC levels. The variance components linkage analysis of the β -globin complex reported here showed that the additive genetic effect from the complex accounted for 42% of the total phenotypic variance in FC levels. The combined linkage and association analysis showed that the *XmnI*- $\text{C}\gamma$ site was responsible for the linkage effect observed at the β -globin complex. This analysis estimated that the combined effects of the sibling common environment and the association with the *XmnI*- $\text{C}\gamma$ site accounted for 32% of the total phenotypic variance in FC levels; a result which is more conservative than the estimate from the variance components linkage analysis and less conservative than the regression analysis. There are clearly some differences in power and efficiency between these various methods of quantifying locus-specific effects on a quantitative trait. This question has yet to be addressed in detail theoretically. Understanding the differences observed in the performance of the different methods will require an extensive investigation.

In conclusion, the results have shown that the *XmnI*- $\text{C}\gamma$ site is highly associated and in strong linkage disequilibrium with high FC levels in a way that cannot be explained by population admixture or stratification. The $\Delta\gamma$ -4 bp deletion site is shown not to be associated with high FC levels and its effect can be explained by the linkage of FC levels to the β -globin complex. Although the *XmnI*- $\text{C}\gamma$ site and the $\Delta\gamma$ -4 bp deletion site are in strong linkage disequilibrium with each other, this does not translate to a significant association between the $\Delta\gamma$ -4 bp deletion site and high FC levels.

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