High-Density SNP Genotyping to Define β-Globin Locus Haplotypes

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Abstract

Five major β-globin locus haplotypes have been established in individuals with sickle cell disease (SCD) from the Benin, Bantu, Senegal, Cameroon, and Arab-Indian populations. Historically, β-haplotypes were established using restriction fragment length polymorphism (RFLP) analysis across the β-locus, which consists of five functional β-like globin genes located on chromosome 11. Previous attempts to correlate these haplotypes as robust predictors of clinical phenotypes observed in SCD have not been successful. We speculate that the coverage and distribution of the RFLP sites located proximal to or within the globin genes are not sufficiently dense to accurately reflect the complexity of this region. To test our hypothesis, we performed RFLP analysis and high-density single nucleotide polymorphism (SNP) genotyping across the β-locus using DNA samples from either healthy African Americans with normal hemoglobin A (HbAA) or individuals with homozygous SS (HbSS) disease. Using the genotyping data from 88 SNPs and Haploview analysis, we generated a greater number of haplotypes than that observed with RFLP analysis alone. Furthermore, a unique pattern of long-range linkage disequilibrium between the locus control region and the β-like globin genes was observed in the HbSS group. Interestingly, we observed multiple SNPs within the *HindIII* restriction site located in the Gγ-globin intervening sequence II which produced the same RFLP pattern. These findings illustrated the inability of

RFLP analysis to decipher the complexity of sequence variations that impacts genomic structure in this region. Our data suggest that high density SNP mapping may be required to accurately define β-haplotypes that correlate with the different clinical phenotypes observed in SCD.

Keywords

single nucleotide polymorphism; β-locus; haplotype; sickle cell disease; Haploview

Introduction

Sickle cell anemia is caused by a SNP in the 6th codon of the β-globin gene, however the disease has great phenotypic heterogeneity and many clinical sub-phenotypes have been described. This observation stimulated research to identify genetic determinants associated with disease severity such as β-haplotypes based on RFLP analysis [1-3]. Five major β-haplotypes designated as Benin, Bantu, Cameroon, Senegal, and India-Arabic based on populations with the designated patterns from different geographical areas, have been associated with SCD [4, 5]. These haplotypes were established with several RFLP sites in the genomic region stretching from the ε-globin to β-globin gene. Among them, the Senegal and India-Arabic haplotypes are generally associated with a benign clinical course in SCD, followed by the Benin haplotype. In contrast, individuals with the Bantu β-haplotype tend to have more severe SCD. These haplotype-phenotype associations are far from perfect, however. In African Americans, the Benin, Bantu and Senegal haplotypes are the major β-locus SNP patterns along with many rare haplotypes. This most likely is due to racial admixture in the African American population [6, 7].

Many attempts have been made to establish haplotype-phenotype relationships in SCD but a robust correlation has not emerged [8-12]. We postulate that the distribution and number of RFLP sites used historically to define β-haplotypes is not sufficient to define the full range of genetic variations in this region. To address this issue we performed high density SNP mapping since it is known that most SNPs do not produce changes in restriction digestion sites however, they could cause DNA structural changes. Recently researchers have begun to utilize new genomic techniques to identify SNPs in genes such as klotho, bone morphogenic protein 6, and annexin A2, whose protein products are related to protection of the cardiovascular system, bone formation, and osteonecrosis respectively in SCD patients [13]. The klotho gene encodes for a transmembrane protein which may protect the cardiovascular system through endothelium derived nitric oxide production. Bone morphogenic protein 6 belongs to transforming growth factor-β superfamily and is associated with growth of bone and cartilage. SNPs have also been identified in other genetic regions and the β-globin gene-like cluster which are associated with elevated fetal hemoglobin levels [14] in sickle cell patients.

A growing number of SNPs have been identified in the β-locus, however the data required to establish β-haplotypes in individuals with SCD is still lacking. Therefore, we believe that SNPs across the β-locus can be used to more accurately define haplotypes that may be associated with the various clinical phenotypes observed in SCD. A chip containing probes

for 88 SNPs in the β-locus region was designed and used for genotyping DNA samples isolated from healthy and SCD African American subjects. Using Haploview analysis, we generated a greater number of haplotypes with the SNP-chip genotypes than with traditional RFLP analysis alone. We also demonstrated that RFLP analysis is not capable of capturing all genomic changes. Furthermore, a unique pattern of long-range linkage disequilibrium (LD) between the locus control region and the β-like globin genes was characterized in HbSS individuals using the SNP-chip approach. These results suggest that high-density SNP mapping is a better approach for defining β-haplotypes which can be used to correlate with the different clinical phenotypes observed in SCD and may have implications for basic research studies in globin gene regulation.

Materials and Methods

Subjects and DNA Isolation

Informed consent was obtained from subjects prior to drawing blood samples in accordance with guidelines of the Institutional Review Boards at the University of Texas at Dallas and collaborating institutions. All samples were obtained from African Americans as follows: eight HbAA and 10 HbSS subjects participated from the University of Texas Southwestern Medical Center (Dallas, TX); 32 samples were obtained from HbSS patients followed in the Sickle Cell Clinic at Howard University (Washington, DC). Twenty-one DNA samples from healthy HbAA individuals were purchased from Coriell Institute for Medical Research (Camden, NJ). All samples were used anonymously. Mononuclear cells were separated from whole blood cells using HISTOPAQUE-1077 separation (Sigma-Aldrich, St. Louis, MO). Genomic DNA was then extracted using Qiagen Flexigene DNA kit (Qiagen Inc., Valencia, CA). Whole-genome amplification was performed using the REPLI-g Kit (Qiagen Inc., Valencia, CA) following the manufacturer's instructions.

RFLP Analysis

Five well-established RFLP sites including the *HincII* site of 5′ ε-globin (5′ε-*HincII*), the *HindIII* sites at intervening sequence II of Gγ-globin (Gγ-IVSII-*HindIII*) and Aγ-globin (Aγ-IVSIII-*HindIII*), *HincII* sites in ψβ-globin (ψβ-*HincII*) and the *HincII* site 3′ of ψβ-globin (3′ψβ-*HincII*) were used to define β-haplotypes by traditional methods. Genomic DNA was first amplified by polymerase chain reaction (PCR) using published primers [15, 16] followed by restriction digestion and agarose gel inspection. The presence of an A or T nucleotide at in the 6th codon of β-globin ($β^6$) was confirmed for all DNA samples using the restriction enzyme *DdeI* and SNP chip analysis. The Gγ-IVSII-*HindIII* region was also sequenced for a subset of DNA samples (8 HbAA and 8 HbSS) to determine the correlation of RFLP digestion patterns with SNPs in this region.

β-Locus SNP-Chip

Eighty-eight SNPs from the β-locus (GenBank: U01317) were used to design a custom SNP-chip in collaboration with Asper Biotechnology (Tartu, Estonia) using arrayed primer extension (APEX) technology [17, 18]. The SNPs summarized in Table 1 were used to perform β-locus SNP genotyping. Briefly, APEX was performed on a two-dimensional array of 60-mer oligonucleotides immobilized on the chip via the 5′ end. DNA samples were PCR

amplified and then hybridized to the chip. Four fluorescently labeled dideoxy nucleotide triphosphates were used for the APEX reaction. The fluorescent signal intensities of the chip was quantified by Genorama™ Genotyping Software (Asper Biotech) and genotypes established for the polymorphic site tested by each probe.

β-Locus Haplotype Analysis

RFLP data were used to construct β-haplotypes based on the ability of the target restriction enzyme to digest (+) or not digest (-) the PCR products generated with gene-specific primers. Since RFLP genotypes are bi-allelic, these data could be used for Haploview 3.2 analysis (http://www.broad.mit.edu/haploview). This software utilizes the estimation-maximization algorithm to calculate D′, the coefficients for LD and to infer haplotypes. To perform Haploview analysis, nucleotide symbols were arbitrarily chosen to represent (+) or (-) pattern for all five RFLP sites. Once the haplotypes were inferred, nucleotide symbols were then replaced with corresponding (+) or (-) signs. By contrast, the SNP-chip genotypes were generated at the nucleotide level and the Haploview analysis was performed without this conversion.

Haploview probes genotypes to determine conformity with Hardy-Weinberg equilibrium. The SNPs with statistically significant departures from the Hardy-Weinberg equilibrium (*p*<0.001) or minor allele frequencies < 5% were excluded from data mining, whereas data for all five RFLP sites were included. SNPs with strong LD (D′ 0.8) were classified into haplotype block using the Four Gamete Rule, a variant of Wang's algorithm [19] and haplotype-tagging SNPs (htSNPs) were selected on a block-by-block basis to identify SNPs that carry non-redundant information about genomic structure.

Results

β-haplotypes were confirmed by RFLP Data

RFLP analysis was performed on genomic DNA samples isolated from 42 HbSS and 29 HbAA individuals for the presence (+) or absence (-) of the five restriction sites in the β-locus as shown in Fig. 1A. Four major β-haplotypes were constructed for HbSS subjects including 39.3% Benin (----+), 22.6% Bantu (-+---), and 3.6% Senegal (-+-++); the Atypical I (-----) haplotype occurred in 16.7% of subjects. The remaining 17.8% of subjects inherited rare or incomplete haplotypes. By contrast, 16 haplotypes were observed in HbAA subjects including 20.9% Atypical II (+----), 18.9% Bantu, 8.6% Benin, and 6.8% Senegal; the remaining twelve haplotypes contributed equally in 44.8% of HbAA subjects. Interestingly, the Atypical II haplotype was not observed in the HbSS group.

We next performed Haploview analysis of the RFLP data (see Materials and Methods for details). This software computes genotypic and allelic frequencies from the input data to infer haplotypes or the combination of alleles at multiple loci that are inherited together on the same chromosome. Haploview analysis identified a subset of the RFLP sites as haplotype-tagging-SNP (htSNPs) that carry non-redundant information and can define the diversity and total number of haplotypes in this region. Using RFLP data we identified 5′ε-*HincII*, Gγ-IVSII-*HindIII*, and Aγ-IVSII-*HindIII* as htSNPs in the HbAA and HbSS study

groups (Fig. 1B and 1C) however the ψβ-*HincII* sites was a htSNP exclusively in the HbAA group. Four major haplotypes were inferred in the HbAA group whereas two major haplotypes were inferred in the HbSS group. By comparing haplotypes generated from the traditional and Haploview analysis, we concluded that less haplotypes were generated using Haploview however a greater number of haplotypes are consistently present in the HbAA subjects. There were also differences on the frequencies of haplotypes. When RFLP analysis was used, the five sites were treated equally, whereas Haploview analysis inferred haplotypes using non-redundant htSNPs indicating that the RFLP sites are not equally informative.

Haploview analysis also allowed us to divide the β-locus genomic region into haplotype blocks defined by SNPs in LD. Fig. 1B and 1C showed there was one haplotype block defined for both groups however for the HbAA group, the haplotype block stretched over a 27 kb region from 5′ ε-globin to ψβ-globin. By contrast, the haplotype block in the HbSS group included a 21-kb region from 5′ ε-globin to Aγ-globin. This data suggest decreased linkage between the RFLP sites in the HbSS group. Based on these observations, we postulated that the genomic structure in this region may have undergone molecular change under the pressure of natural selection.

Unique haplotype patterns are identified in the β-locus by high-density SNP mapping

We next determined whether SNP-chip genotyping with more dense coverage across the β-locus would reveal greater differences in genomic structure within the two study groups than that produced by RFLP analysis. To achieve this end, we designed a custom SNP-chip based on APEX technology (see Materials and Methods). Because the globin genes are highly homologous and there exists many repetitive sequences throughout the locus, we were only able to identify 88 SNPs that could be reliably tested by APEX technology (Fig. 1A). The same DNA samples used in the RFLP analysis were genotyped using the SNP-chip. The Gγ-IVSII-*HindIII* site was included on the chip to retest the SNP at position 35770 (C/G) to confirm our RFLP data.

Genotypes for DNA samples isolated from 29 HbAA and 42 HbSS African Americans were generated using the SNP-chip. Haploview analysis identified 48 SNPs with minor allele frequency <5% in the HbAA group, while 61 SNPs had minor allele frequency <5% in the HbSS groups; three additional SNPs (rs9736333, rs3759071 and rs6578593) deviated from the Hardy-Weinberg Equilibrium in the HbSS group. After these exclusions, there were 40 and 24 SNPs included in the HbAA and HbSS groups respectively for further analysis to determine LD and major haplotype patterns in the β-locus.

The first procedure completed with the genotype data was the generation of "heat maps" as shown in Fig 2A and 2B. Each column in the heat map represents individual samples, while each row represents one SNP. The genotypes for the SNPs were color coded as follows: blue representing homozygous wild type; red for homozygous mutant alleles and yellow for heterozygous alleles. Note for some SNP positions there were samples for which genotyping data was not generated (white boxes). From these maps, we concluded that genotypes alone were not as meaningful. However, when individuals with similar genotype patterns were grouped together, then the heat maps revealed different genotype patterns for the two study

groups as a whole. For the HbAA group, the appearance of heterozygous genotypes is very diverse and random compared to that observed for HbSS subjects. These findings suggest a dramatic difference in the β-locus genomic structures for the two groups. This difference was not observed with the RFLP genotypes (data not shown). We concluded that the β-locus in healthy subjects had a higher frequency of recombination events that that observed in the HbSS population.

We next performed a detailed analysis of the SNP-chip data using Haploview software. A pair-wise computation between SNPs alleles and genotype frequencies was completed to determine the degree of LD across the β-locus to identify htSNPs, and to define the haplotype blocks and inferred β-haplotypes. We first constructed the major haplotypes across the entire β-locus for comparison with RFLP analysis by calculating the linkage between haplotype blocks or multi-allelic D′ (Fig. 2C and 2D). Nine haplotype blocks were defined for the HbAA subjects that produced 42 haplotypes including six at a frequency from 3.4-5.1% and 36 minor haplotypes accounting for 76.4% of the genetic variation in this group. A similar analysis of the HbSS subjects showed five haplotype blocks as shown in Fig. 2D. By contrast, 21 haplotypes were defined in the β-locus including four major haplotypes at a frequency of 40.4%, 13.1%, 10.7% and 5.9% account for 70.1% of genomic variation. Finally, we identify 28 htSNPs for the HbAA and 16 htSNPs for the HbSS subjects (Fig. 2C and 2D, indicated by inverted triangles) that are sufficient to define the major haplotypes in each group. When these data were compared to the RFLP analysis, there was no correlation between haplotypes generated by the two methods. The SNP-chip results clearly demonstrated that a greater number of haplotypes and differences in the genomic structure of the β-locus exist between the HbAA and HbSS study groups which were not identified by RFLP analysis.

Genomic complexity in the Gγ-IVSII-*HindIII* site demonstrated by SNP-chip analysis

For SNP-chip analysis, we targeted the C to G (C/G) SNP at nucleotide 35,770 which destroys the *HindIII* site (AAG**C**TT) in the Gγ-globin intervening sequence II region. However, the SNP-chip genotypes generated were not in agreement with the RFLP results for the Gγ-IVSII-*HindIII* digestion pattern. We observed a discrepancy in that the RFLP result was (+- or --) in the presence of a CC genotype obtained from SNP-chip results. Since the *HindIII* site has been commonly used in RFLP studies to define β-haplotypes, we believed that direct sequencing was necessary to determine the basis of the inconsistent results. Sixteen samples from 8 HbAA and 8 HbSS subjects were sequenced for this region. The results are summarized in Fig. 3. At position 35,770 all samples had a homozygous CC genotype. Interestingly, two additional SNPs at positions 35,769 and 35,772 were identified that correlated with the RFLP digestion patterns. The SNP at 35,769 has not been reported, but the SNP at 35772 was registered in the dbSNP database (rs2070972; G/T).

The sequencing data showed that HbAA subjects had GG genotypes at 35,769 whereas HbSS subjects were heterozygous GT. For the SNP at 35,772 there was greater variability in the HbAA group however the HbSS subjects were all heterozygous GT. By comparison with the RFLP digestion patterns and the predicted effect on *HindIII* digestion, for HbAA subjects the 35,772 SNP was predictive of the RFLP result (-- or +-) produced by a variable

sequence of AAGCT[T/G]. For the HbSS samples, two RFLP patterns were observed which correlated with heterozygosity at position 35,769 or 35,772 (AA[G/T]CT[T/G]). The sequencing and digestion data combined showed the association of AA<u>T</u>CT<u>G</u>, and AA<u>T</u>CT<u>T</u> DNA sequence with the HbSS subjects. It remains to be addressed whether these variations can be correlated with the various clinical phenotypes observed in SCD. Mutations in the intervening sequences in other β-like globin genes have been associated with various hemoglobinopathies [20, 21] and γ-gene expression in K562 cells [22]. Our results demonstrated that *HindIII* digestion alone could not accurately distinguish the underlying genomic structural differences at the nucleotide level in the Gγ-globin gene.

High-density β-locus genotyping reveals unique htSNPs in HbSS subjects

In our next analysis, we compared the frequencies of the associated alleles for 22 SNPs from both groups. As shown in Table 2, there were only two SNPs, rs6578593 and rs7480526, which occurred at similar frequencies in both groups. Thirteen SNPs including six in the hypersensitive sites, four in ε-globin, two in γ-globin (rs10128653 in Gγ- and rs11827654 in Aγ-globin) and one in ψβ-globin showed higher allele frequency in the HbSS group. Seven SNPs displayed the opposite patterns with higher frequency in HbAA subjects. With the vast number of SNPs identified, it is beneficial to identify htSNPs that can be used to capture the major haplotype variants without analyzing all SNPs in a given region. Interestingly we discovered three SNPs that were unique to either the HbSS or HbAA group indicating different patterns of inheritance for the two groups. Identifying specific htSNPs will be useful for population studies or characterization of clinical sub-groups in SCD patients.

In agreement with the diverse genotypes and high number of (β-haplotypes in the HbAA group, nine haplotype blocks were generated and weak LD was observed in this group demonstrated by white boxes across the (β-locus (Fig. 4A). However, we observed five haplotype blocks in the HbSS group as shown in Fig. 4B. In contrast to the weak LD in the HbAA group, very strong LD was exhibited in the HbSS group between SNPs in the locus control region and several downstream SNPs located in ε-globin (rs7479652, rs3759069, rs4910740 and rs11820733), Gγ-globin (rs10128653 and rs2855122), Aγ-globin (rs11827654, rs2402330), ψβ-globin (rs2071348), and the β-globin gene (rs7480526) which is illustrated by the stretch of red boxes across the (β-locus region. The locus control region is required for developmental stage-specific globin gene expression through interaction with downstream promoter regions [4, 23-25]. Of note, rs2855122 was recently shown to be involved in drug-mediated Gγ-globin fetal hemoglobin induction by butyrate [26]. Thus, strong LD between these SNPs may affect the genomic structure of this region, which may contribute to the regulation of γ-globin gene transcription.

Haploview analysis of SNP-chip data also allowed us to establish the LD patterns across the entire (β-locus for both study groups. In Fig. 2C and 2D, the values for multi-allelic D′ are shown between haplotype blocks; LD between blocks with a D′ 0.8 is indicative of strong linkage. We observed weak linkage across the β-locus of HbAA subjects (mean multi-allelic D′=0.69) in contrast to strong linkage in the HbSS subjects (D′=0.84). This suggests that the presence of β^S mutation and natural selection pressure had a long-range effect on the locus control region and downstream β-like globin genes.

Discussion

Historically, RFLP analysis has been used as the preferred approach to establish β-haplotypes. Our results showed that three of the most commonly used RFLP sites were identified as htSNPs in the HbSS group suggesting that the five RFLP sites are not equally informative. Furthermore, these sites are located in region of moderate LD in the HbSS group. We also demonstrated that three SNPs in the Gγ-IVSII-*HindIII* site produced variable RFLP readouts based on genotypes which could affect the function of this intronic region and Gγ-globin transcription rates. Furthermore, a greater number of haplotypes were inferred from the SNP-chip data than with RFLP analysis, which could be used to define various sub-phenotypes. It has been suggested that genotyping a few widely spaced sites may result in missing important genomic variations [27]. Thus, our data demonstrate that the RFLP approach is not sufficient to reveal the genomic complexity of the β-locus and that high-density SNP mapping using new technology is preferred to discover a wide range of genetic determinants.

Despite the fact that HbSS is caused by a single A/T mutation in the sixth codon of the β-globin gene there are many different clinical phenotypes associated with this monogenic disorder. Patients with elevated fetal hemoglobin levels generally have less severe disease. Research efforts to identify epigenetic factors that alter γ-gene transcription synthesis have shown that SNPs in the β-locus or other distant genomic regions contribute to γ-globin regulation. Mutations including the -158 *XmnI* (C/T) SNP in the Gγ-globin gene promoter [28, 29] and distant SNPs in the 6q22.3-q23.2, 8q11-q12 and Xp22.2-p22.3 regions have been associated with elevated fetal hemoglobin levels [30-32]. It is believed that other genetic markers in the β-locus also exerted a significant effect on fetal hemoglobin synthesis [33].

To determine the correlation of SNPs in the β-locus with phenotypic outcomes in SCD, we constructed a SNP chip containing 88 SNPs across this region to study the LD of SNPs in the two study groups. SNP genotyping revealed differences between the healthy HbAA and HbSS subjects which were not observed by RFLP analysis. The healthy group showed diverse haplotype patterns with moderate LD over a 35 kb region but weak LD over the entire β-locus. By contrast, the HbSS group showed strong LD between SNPs stretching over 60 kb. A recent publication also described strong long-range linkage across the β^S mutation in African and Afro-Caribbean HbSS populations [34].

Thus, our observations demonstrate that the traditional RFLP approach does not accurately reflect the complexity of the β-locus. Our study is the first detailed genomic analysis of this region in African Americans using modern genomics techniques. SCD is increasingly viewed as a disease whose phenotype is affected by an array of modifier genes. The clinical manifestations of SCD are most likely a result of variations on the β-locus in addition to other genomic regions. Therefore, high-density β-locus and genome-wide SNP mapping with a greater sample size should impact efforts to ascertain genotype-phenotype relationship in SCD.

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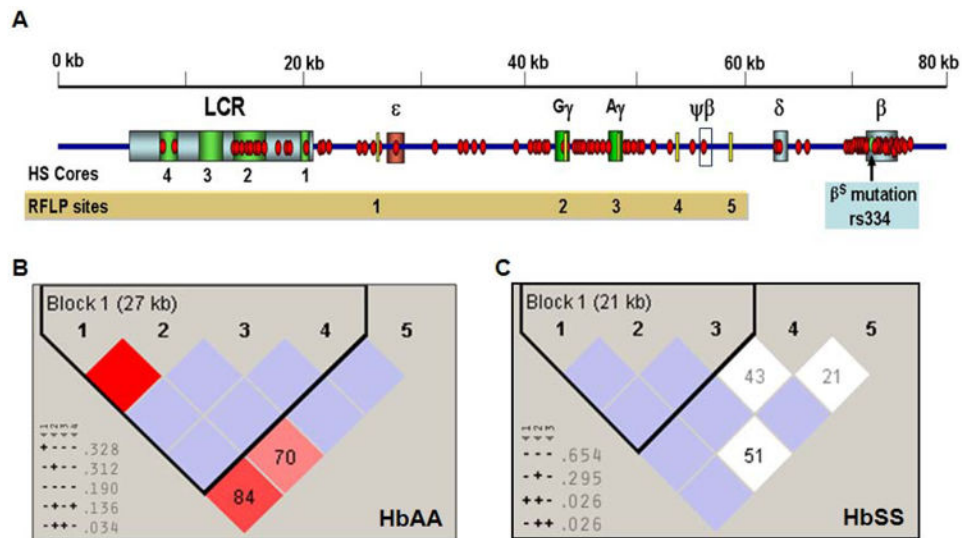36. A haplotype map of the human genome. Nature. 2005; 437:1299–320. [PubMed: 16255080]

Fig. 1. β-haplotypes established using RFLP genotypes
A) Schematic diagram showing the distributions of 88 SNPs (red dot) on the custom SNP-chip and five RFLP sites (yellow bar) that were analyzed. Each globin gene is indicated by a colored box and ψβ-globin is shown by a white box. The β^S mutation (rs334) is shown as a green circle. Abbreviations: LCR, locus control region; HS, DNase I hypersensitive site. **B)** The linkage disequilibrium (LD) patterns and haplotypes established by Haploview analysis for the RFLP genotypes are shown for the HbAA subjects. Pair-wise computation was performed for the SNPs which are shown on each side of the boxes. The degree of LD is defined by value of D′ and LOD (the logarithm of the likelihood odds ratio), which is a measure of the confidence of D′ values. The red boxes indicate strong LD (LOD >2, D′ = 1), white boxes no LD (LOD < 2, D′ < 1), and pink (LOD = 2, D′ <1), and blue (LOD < 2, D′ =1) boxes indicate intermediated LD. The D′=1 unless indicated in the boxes where D′ is multiplied by 100. SNPs with strong LD were defined into haplotype blocks (black triangle) and the size of the region in LD is shown in parentheses. The inferred haplotypes and frequency observed are shown in the lower corner; htSNPs are indicated by a gray triangle beneath the RFLP number. Symbols: "+" = cut with the specific restriction enzyme used for analysis; "-"= no cut. **C)** The LD patterns and haplotypes for the RFLP genotypes established by Haploview analysis are shown for the HbSS group. The symbols are the same as defined in Panel B.
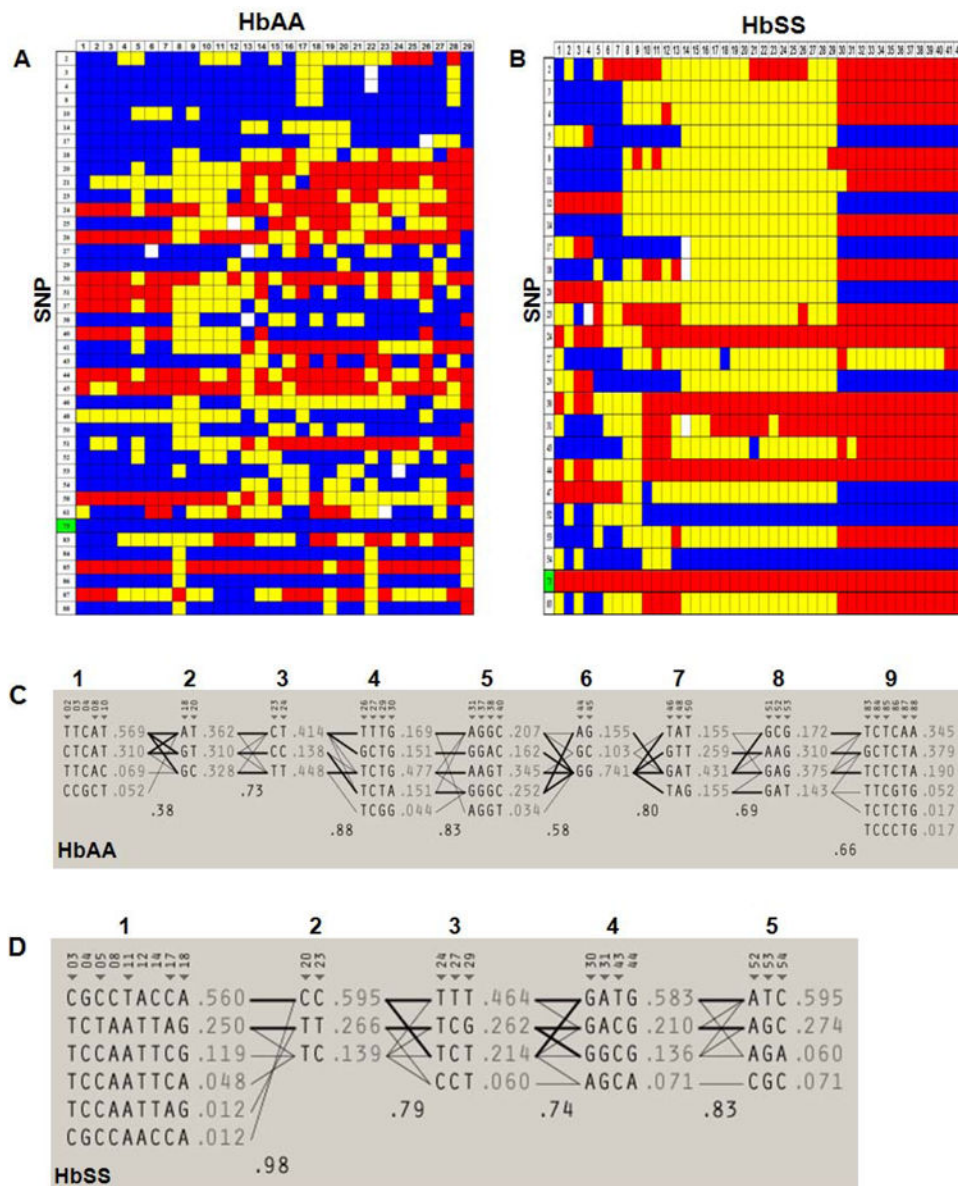
Fig. 2. Haploview-inferred haplotypes using SNP-chip genotypes

A) A genotype heat map was established for the HbAA subjects. Genotypes produced using the SNP-chip that satisfied the criteria detailed in Materials and Methods are shown. The rows represent the SNPs analyzed and the columns represent each DNA sample tested. SNPs are numbered according to that defined in Table 1; the β^S SNP (rs334) is number 73 and is highlighted in green. At each SNP position, a blue box represents wild-type homozygous genotypes, a yellow box heterozygous and the red box homozygous mutant genotypes. White boxes indicate genotype data is missing for the SNP indicated. **B)** Genotype heat map for the HbSS subjects. The color code is the same as described in Panel A. **C)** Haploview software was used to infer haplotypes using SNP-chip genotype data for the HbAA subjects. The numbers in the gray shaded area represent the SNPs listed in Table 1. The inferred haplotypes in each haplotype block and linkage between blocks are shown.

Nine haplotype blocks were defined (numbers above the gray shaded area). The lines between haplotype blocks indicate the linkage frequency with a thick line for > 10% and a thin line for a frequency from 1-10%. Haplotype-tagging SNPs (htSNPs) are indicated underneath the SNP numbers by a triangle (▼). Haplotype frequency within each haplotype block is shown next to each haplotype. The number between two blocks is the Hedrick's multi-allelic D′; D′ >= 0.8 is indicative of strong LD between the blocks. **D)** Inferred haplotypes and linkages between five haplotype blocks (numbers above the gray shaded area) for the HbSS subjects. The methods and symbols are the same as described in Panel C.

Fig. 3. Correlation of *HindIII* digestion with direct sequencing of the Gγ-IVSII region
The PCR products generated for RFLP analysis was used for direct sequencing analysis. The *HindIII* restriction site **AAGCTT** and position of three nucleotides according to the HBB record (U01317) are shown at the top of the figure. Each row represents data for individual DNA samples; each nucleotide position and genotype is shown for both alleles based on the sequencing results. For RFLP patterns the positive symbol (+) refers to *HindIII* digestion, while the negative symbol (-) no cut.
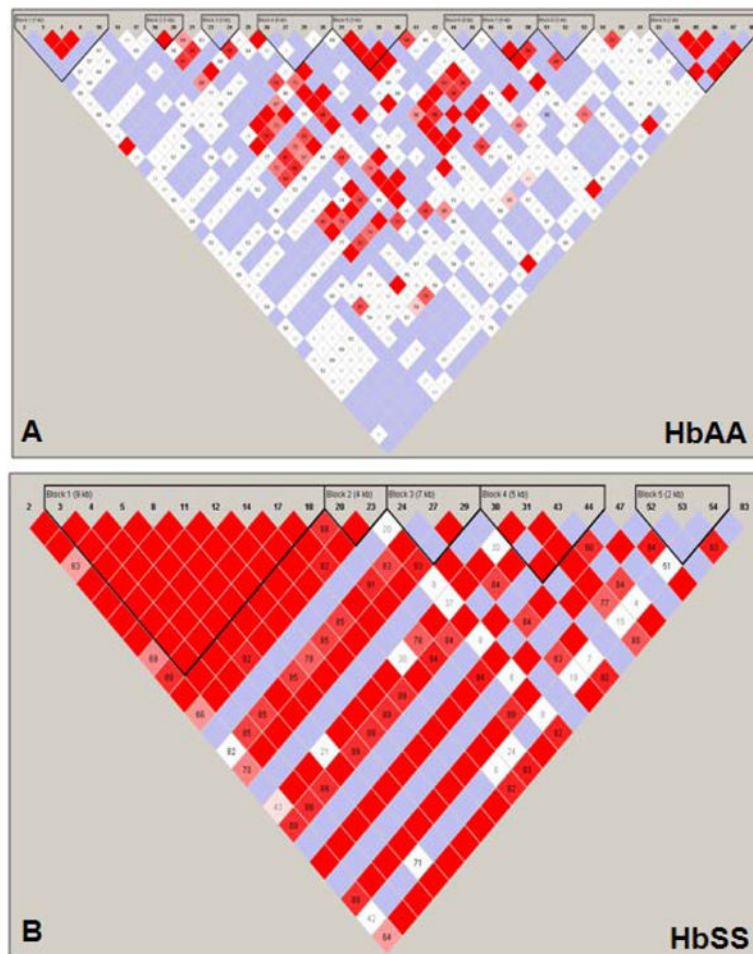
Fig. 4. Linkage Disequilibrium and haplotype block patterns across the β-locus
A) Shown are the LD patterns and haplotype blocks established by Haploview analysis with the SNP-chip data for the HbAA group. The numbers on top represent the SNPs used for the analysis. Haplotype blocks are shown by inverted triangles. Within each haplotype block, the distance of the region covered is indicated in kilo-bases (kb). The color code is the same as defined in the legend for Fig. 1B. **B)** The LD patterns and haplotype blocks established by the Haploview analysis with the SNP-chip genotype data for the HbSS group are shown. The symbols are the same as defined in Panel A

Table 1

Summary of Single Nucleotide Polymorphisms on the β-Locus SNP Chip

No.	ID	Pos.	SNP
LCR			
1	rs11826674	1270	T/G
2	rs7119142	1580	T/C
3	rs34272388	7573	T/C
4	7579	7579	C/G
5	rs12292063	8254	C/T
6	8365	8365	A/C
7	8384	8384	A/G
8	rs7119428	8580	A/C
9	rs9736333	8598	A/G
10	rs10837757	8616	T/C
11	9114	9114	A/T
12	rs7936221	10463	A/T
13	rs4638332	10494	G/T
14	rs7946623	12346	T/C
15	rs11036587	13372	G/A
16	rs11036586	13393	G/A
17	rs11036571	15024	C/A
ε-globin			
18	rs7479652	17554	G/A
19	rs3759068	18633	A/G
20	rs3759069	18831	C/T
21	rs3759071	19130	T/C
22	rs2213165	20983	C/G
23	rs4910740	23370	T/C
24	rs10160271	25990	C/T
25	rs11036507	26351	A/G
26	rs10160678	27102	G/T
27	rs11820733	29227	C/T
28	rs5010978	31343	C/A
Gγ-globin			
29	rs10128653	33028	T/G
30	rs2855121	33198	A/G
31	rs2855122	33253	G/A
32	rs3020750	34154	T/C
33	rs2860456	34161	G/A
34	34163	34163	A/C
35	34874	34874	G/A
36	35770	35770	C/G
37	rs2236794	36224	G/A
38	rs10768707	36570	G/A
39	rs2855032	36786	C/T
40	rs2255519	36952	C/T
41	rs2855126	37346	G/C
42	rs2855034	37486	C/T
Aγ-globin			
43	rs11827654	37975	C/T
44	rs2855039	38826	A/G
45	rs2855040	39045	C/G
46	rs28440105	40688	G/T
47	rs2402330	41022	T/A
48	rs916111	41144	A/T
49	rs916112	41209	A/T
50	rs6578592	41347	T/G
51	rs7924684	43756	A/G
ψβ-globin			
52	rs2071348	46334	A/C
53	rs11036415	47670	G/T
54	rs10488677	48982	C/A
δ-globin			
55	54886	54886	G/A
56	55047	55047	G/T
57	rs3752382	56514	C/T
58	rs7112844	58260	A/T
β-globin			
59	61144	61144	G/A
60	rs12289247	61413	G/T
61	rs11036364	61434	T/C
62	rs10742584	61668	T/C
63	rs10742583	61797	T/C
64	62027	62027	G/A
65	62036	62036	C/T
66	rs33944208	62049	C/T
67	rs33941377	62050	C/G
68	rs33980857	62107	T/A
69	rs34598529	62108	A/G
70	62153	62153	C/?
71	62160	62160	G/A
72	62166	62166	T/C
73	**rs334**	**62206**	**A/T**
74	rs33926764	62211	A/G
75	rs35799539	62219	C/?
76	62225	62225	T/C
77	rs35890959	62247	G/A
78	rs35724775	62284	T/C
79	rs11549407	62434	A/C
80	rs11549406	62560	C/G
81	rs1803195	62567	G/T
82	rs10768683	62647	C/G
83	rs7480526	62705	G/T
84	rs7946748	62712	C/T
85	rs1609812	63297	C/T
86	rs12788013	63843	C/G
87	rs10837631	64081	A/T
88	rs10768682	64928	A/G

The 88 SNPs included on the SNP-chip are listed. Nucleotide positions (Pos.) were established using the HBB record U01317 and distribution of SNPs across the β-globin locus are indicated at the beginning of each section. The $β^S$ SNP, rs334, is indicated in bold. The column labeled "SNP." contains the wild-type/polymorphic nucleotide. dbSNP ID is used to identify a SNP in the database based on dbSNP build 127. Position numbers in the HBB record for SNPs not in the dbSNP database were used as unique identifiers. Abbreviations: LCR, locus control region.

Table 2

SNP Frequency and htSNPs in the HbAA and HbSS groups

No.	ID	Position	SNP	Associated Allele	Frequency		HbAA htSNP	HbSS htSNP	β-Locus Region
					HbAA (%)	HbSS (%)			
2	rs7119142	1580	T/C	C	36.2	76.2			**HS4**
3	rs34272388	7573	T/C	C	5.4	57.1			
4	7579	7579	C/G	G	5.4	57.1			**HS2**
8	rs7119428	8580	A/C	C	5.2	57.1			
14	rs7946623	12346	T/C	C	12.1	57.1			
17	rs11036571	15024	C/A	A	8.9	25.6			**HS1**
18	rs7479652	17554	G/A	A	36.2	62.2			
20	rs3759069	18831	C/T	C	32.8	59.5			
21	rs3759071	19130	T/C	T	31	46.4			**ε-globin**
23	rs4910740	23370	T/C	T	44.8	25.6			
24	rs10160271	25990	C/T	C	13.8	6			
27	rs11820733	29227	C/T	T	16.7	46.4			
29	rs10128653	33028	T/G	G	5.2	26.2			
30	rs2855121	33198	A/G	A	15.5	7.1			**Gγ-globin**
31	rs2855122	33253	G/A	G	41.4	20.7			
43	rs11827654	37975	C/T	T	24.1	58.3			**Aγ-globin**
44	rs2855039	38826	A/G	A	15.5	7.1			
46	*rs6578593	40688	G/T	T	31	36.9			
52	rs2071348	46334	A/C	C	17.2	7.1			**ψβ-globin**
53	rs11036415	47670	G/T	T	14.3	59.5			
54	rs10488677	48982	C/A	A	15.5	6			
73	**rs334**	**62206**	**A/T**	**A**	**100**	**0**			**β-globin**
83	*rs7480526	62705	G/T	G	37.9	33.3			

The allele frequency for 23SNPs including some htSNPs ( ) for the HbAA and HbSS groups are summarized. SNP selection criteria are described in the text. The β^S SNP (rs334) is indicated in bold letters. The column labeled "Variation" refers to wild-type/polymorphic nucleotide. The frequency was calculated for the associated allele. The "*" indicates SNPs showing similar allele frequencies for both group. The nucleotide positions were located using the HBB record U01317; SNP distribution across the β-globin locus is indicated. Abbreviations: HS, hypersensitive site.