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Genetic diversity and linkage disequilibrium in the chemokine receptor *CCR2-CCR5* region among individuals and populations

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Abstract

Background—Chemokine receptors *CCR2* and *CCR5* play a key role in immune and inflammatory responses and have been associated with several diseases, including AIDS. In order to comprehend health disparities it is important to understand the nature of genetic variation in specific genes of interest in different populations. Current studies of the *CCR2* and *CCR5* receptor genes are primarily focused on the *CCR5*-32, and *CCR2*-V64I SNPs.

Methods—Sanger sequencing was used to sequence the regions containing 16 SNPs in the adjacent *CCR2* and *CCR5* genes (including *CCR5*-32, and *CCR2*-V64I) in 249 subjects of African, European and Hispanic ancestry. Linkage disequilibrium (LD) and haplotypes were determined using Haploview.

Results—The data revealed large differences in allele frequencies of several SNPs and LD patterns among the ethnic groups, including SNPs that were restricted to Africans or Europeans. Seven known *CCR5* haplotypes and six novel *CCR2* haplotypes were identified. A rare case of an HIV+ subject with the *CCR5*-32/32 was identified.

Conclusions—These data demonstrate a LD between *CCR2* and *CCR5* at several loci and provide new information about *CCR2* that contributes to our understanding of its population-specific genetic variability. The data indicate that in addition to *CCR5*-32 and *CCR2*-V64I, other SNPs and haplotypes may be important genetic determinants of disease and should be investigated.

Keywords

CCR5; *CCR2*; ethnicity; health disparities; chemokine receptors; HIV; AIDS

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1. INTRODUCTION

Chemokines act on immune cells by binding to G-protein-coupled C-C chemokine receptors (CCRs). CCRs have been noted to play an important role in immune and inflammatory responses and regulate several disease states including viral infections. Most of the human C-C chemokine receptor genes form a gene cluster on chromosome 3p21-22. Receptors CCR1-5 are very similar in structure suggesting that they originated through gene duplications. *CCR2* and *CCR5* are adjacent genes that are more closely related than the other members of the cluster based on sequence similarity. Polymorphisms in these two genes have been associated with varying levels of vulnerability to a number of diseases, including atherosclerosis, cervical cancer, asthma and most notably HIV-1 [1–5].

A substitution of an isoleucine for a valine at position 64 in the *CCR2* gene (V64I) has been associated with delayed progression of HIV to AIDS [5–8]. A point mutation in the regulatory region of the *CCR5* gene (rs1800024; –1835C>T) that was associated with slow AIDS progression, is in linkage disequilibrium (LD) with the *CCR2*-V64I variant [7, 9, 10]. Because the V64I polymorphism is located in a highly conserved region of *CCR2*, its functional effects may be related to linked SNPs in *CCR5* [6]; however little is known about its relationship with other *CCR2* SNPs.

A frameshift mutation due to a 32 base pair deletion in *CCR5* (*CCR5*- 32; rs333) that encodes a truncated protein is reported to reduce susceptibility to HIV-1 M tropic strains [11–13]. 32 results in a European-specific, non-functional receptor and is purported to be protective against HIV-1 infection. A variant in the *CCR5* promoter (rs1799887; –2459A>G), has been reported to result in delayed HIV to AIDS progression [14].

There is also evidence that SNPs and haplotypes of the *CCR5* and *CCR2* genes differ in frequency and function between populations [15–18]. A well-studied *CCR5* haplogroup (HHF) that is linked to the *CCR2*-V64I variant shows different frequencies and functions across populations [16, 19]. Resistance to HIV infection was associated with the *CCR5*- 32 deletion and related to the HHG*2 haplotype; however this deletion has low frequency (0.1%) among African populations [16, 20].

In addition, *CCR5* diplotypes with HHA or HHC haplotypes were associated with variable effects on disease progression. When paired with the *CCR5*- 32 (HHG*2) deletion bearing haplotype, the HHG*2/HHA and HHG*2/HHC diplotypes have been associated with disease retarding effects and HHE/HHG*2 was associated with HIV disease acceleration [21]. However, aside from the relationship described between the *CCR2*-V64I SNP and several *CCR5* haplotypes, there is little information regarding frequencies of other *CCR2* SNPs and haplotypes [20].

This study aims to explore and compare the haplotype structure of the *CCR2*-*CCR5* region in subjects of African, European and Hispanic ancestry.

2. MATERIALS AND METHODS

2.1 Samples

Samples were obtained as a part of two other studies [22–23]. Postmortem brain samples (n=14 HIV-1 negative; n=24 HIV-1 positive) and peripheral blood mononuclear cells (PBMC) samples (n=116; all HIV-1 positive) were obtained from The Manhattan HIV Brain Bank, Mount Sinai School of Medicine, New York, NY [24]. An additional set of PBMC samples (n=95) from healthy volunteers (exclusion criteria are described elsewhere [25]), were obtained from the Laboratory of the Biology of Addictive Diseases Rockefeller

University, New York as a control group. Ethnic categories were assigned based on self-report. The following categories were included: African ancestry (AF), European ancestry (EU) and Hispanic ancestry (HI).

2.2 DNA Preparation, SNP Selection, Genotyping and Sequencing

DNA was isolated from postmortem brain and PBMC using the Easy-DNA™ Kit (Invitrogen, Carlsbad, CA, USA). The oligonucleotide PCR primers used to amplify *CCR5* (NM_000579.3) and *CCR2* (NM_001116513.2) are listed in Supplementary Table S1. SNPs were selected based on minor allelic frequencies greater than 5% in at least one population, proximity to other SNPs, and/or the SNP was discussed in the literature. *CCR5* SNPs were chosen based on previous reports from Gonzalez et. al. [16] and Picton et. al. [19]. Primers for SNP #16 were taken from another study [26]. Platinum PCR Supermix (Invitrogen, Carlsbad, CA) was used for PCR reactions with the following cycling conditions: 94° C, 10 min; 94° C, 30 sec; 63° C, 30 sec; 57° C, 30 sec; touchdown 1° C, 8 cycles; 94° C, 30 sec; 56° C 30 sec; 52° C, 30 sec; 35 cycles; 72° C, 7 min, 4° C. Amplicons were treated with ExoSAP-IT® (USB Corporation, Cleveland, OH). Amplicons were sequenced using ABI BigDye Terminator v3.1 Cycle Sequencing Kit and run on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). Chromatograms were analyzed using Sequencer 4.9 (Gene Codes, Ann Arbor, MI). There was less than a 10% genotype failure rate for any SNP, however in most cases adjacent SNPs provided enough information to predict haplotypes.

To confirm previously reported SNP frequencies in *CCR5* [16, 19], we used a representative subset of samples from the three ethnic groups (n=38). To determine unique patterns not previously reported for *CCR2*, all samples were genotyped. The *CCR2* sequence analysis was performed in two steps: the first step was performed in 38 subjects (cohort 1), and the second step was performed in an extended group of 211 subjects (cohort 2). We compared and confirmed our results to allele frequencies published in several databases (HapMap, SNP500, ALFRED; <http://alfred.med.yale.edu>, and the 1000 Genomes Project).

2.3 Linkage Disequilibrium and Haplotypes

Haploview v 4.2 was used to determine the extent of linkage disequilibrium (LD). LD blocks and haplotypes were identified independently for each ethnicity and also for the whole group. LD was calculated using the Pearson correlation test (r^2). Haplotypes were built using custom defined blocks to include all SNPs genotyped. We assigned numbers to each *CCR2* haplotype (H1-H6). *CCR5* Haplotypes were categorized based on nomenclature described by Gonzalez et al. [16].

3. RESULTS

3.1 Allele frequencies

In the first part of this study, the 200 bp regions around eight SNPs of the *CCR2* gene and eight SNPs of the *CCR5* gene were genotyped using Sanger sequencing (Table 1; Figure 1). The commonly studied variant *CCR2*-V64I (rs1799864) is indicated as SNP #4 on *CCR2* exon 2, and *CCR5*- 32 is labeled as SNP #16 on *CCR5* exon 3.

There were no significant differences in allelic frequencies between subjects that were HIV-1 positive or negative, therefore each ethnic group was analyzed as a whole. Large differences in allele frequencies were found among the three groups (African, European and Hispanic); (SNP #s 2–4, 6–9, and 13–16). Notably, SNP #6 (rs3918386) and #13 (rs41469351) were only found in subjects of African ancestry, and SNP #16 (rs333, 32) was only found in subjects of European ancestry (Table 1).

To compare our result to other studies, allele frequencies were obtained from several public databases. Notably, while the *CCR2*-V64I (SNP #4) had the most frequency data available across databases, frequency data on *CCR2*-SNPs # 2, 3 and 6, and *CCR5*-SNPs # 9 and #13 were minimal in some of the databases selected for comparison. Furthermore, SNP frequency information on the specific Hispanic population used in our study was lacking for both *CCR2* and *CCR5*, therefore we used the Puerto Rican (PUR subgroup in 1000 Genomes Project) when available. All other SNP frequencies were compatible with our findings (Table 1).

3.2 CCR5

LD analysis of *CCR5* SNP #s 9–16 revealed that SNP #s 10–14 form an LD block in Europeans (Figure 2A). SNP #11 (rs1799987), which was identified as a protective allele across multiple ethnic groups [14] is in complete LD with SNP # 12 in Africans and Hispanics (Figure 2B and 2C).

Seven *CCR5* haplogroups were identified, with high compatibility in frequencies to those described by Gonzalez [16] (Table 2). HHC represents the most frequent haplotype in Europeans and Hispanics. HHA and HHD are most frequent in Africans. HHG*2 is the only haplotype associated with *CCR5*- 32 (SNP #16). HHB was not identified in our sample. *CCR5* diplotypes are shown in Supplementary Table S2.

3.3 CCR2

The *CCR2* sequence analysis was performed in two steps in two cohorts (see methods). . There was no significant difference between the findings in the two cohorts, so they were combined for haplotype analysis. Since SNPs #2, #3 and #7 were shown to be in high LD in the first cohort, SNPs #2 and #3 were excluded from analysis of the second cohort.

LD analysis in cohort 1 revealed that the European sample have the greatest number of SNPs in complete LD ($r^2 = 1$): SNPs #1, #2, #3, #5 and #7, as well as SNPs #4 and #8 (Figure 2A). In contrast, the African sample have the smallest number of SNPs in complete LD: SNPs #2, #3 and #7 (Figure 2B). The Hispanic sample have fewer SNPs in complete LD than the European but more than the African sample: SNPs #4 and #8, #1 and #5, as well as #2, #3 and #7 (Figure 2C).

Six major haplotypes (frequency greater than .03) were identified in *CCR2* (labeled H1-H6) (Table 3). Of these six haplotypes, we identified a major haplotype (H1), which was represented in 67% of subjects across the three ethnic groups. In addition, H5 and H6 are only found in the Africans and Hispanic samples. Notably, the V64I variant is only represented in haplotype H3 (Table 3).

Diplotype H1/H1 was the most common diplotype among the three ethnic groups (Table 3 and Supplementary Table S3). While the H1/H5 diplotype was most frequent in the African sample, it was not found in the European sample. The Africans sample had most diplotype patterns, excluding H3/H3. The European sample had fewer than 55% of the diplotype patterns and diplotypes that contained H5 or H6 were not represented (Table 3 and Supplementary Table S3).

3.4 CCR2/CCR5

LD between *CCR2* SNPs and *CCR5* SNPs were determined in cohort 1. Several relationships between SNPs in the two genes were identified: *CCR2* SNP #4 (V64I) and *CCR5* SNP #15 are in complete LD ($r^2=1$) in all subjects. *CCR2* SNP #8 and *CCR5* SNP #15 are in complete LD in Europeans only (Figure 2B). SNPs *CCR2*-SNP #6 and *CCR5*-

SNP #13, that were found only in the African and Hispanic groups are in complete LD (Fig 2A and 2C). *CCR2* haplotype H3 (the only haplotype with the V64I variant/SNP 4) is associated with the *CCR5* HHF haplotype (Supplementary Table S4) defined by Gonzalez [16] as HHF*2. None of the V64I carriers had the $\Delta 32$ mutation. *CCR5* $\Delta 32$, which is carried on haplotype HHG*2, was associated with *CCR2* haplotypes H1 and H4 (Supplementary Table S2 and S3).

3.5 *CCR5*- $\Delta 32$

Interestingly, a 33-year old European male subject (Subject MHBB 10015) was identified who was homozygote for *CCR5* $\Delta 32$ but was HIV seropositive (Supplementary Figure 1). The subject was diagnosed with HIV-1 in 1991 and died in 1999. His HIV viral load was measured as 176800 copies/mL and CD4⁺ T-cell count was 66 cells/mL at 6 months before death. This subject had a homozygous genotypic profile at each of the 16 alleles genotyped (*CCR2* and *CCR5*). He was homozygous for the *CCR5* HHG*2 haplotype and the *CCR2* H1/H1 haplotype. He did not carry the *CCR2*-V64I variant nor the *CCR5* promoter variant rs1799987/SNP #11 (Supplementary Table S2 – S3).

In addition, we identified another European male (MHBB 10003, 45 years old) and a Hispanic female (MHBB 10045, 31 years old) who were also HIV positive, and heterozygous for *CCR5* $\Delta 32$. Similarly to Subject 10015, Subject 10003 has the *CCR2* H1/H1 diplotype but a *CCR5* HHG*2/HHC diplotype. We were unable to obtain his viral load and T-cell count. Subject 10045 had the *CCR2* H1/H4 diplotype and the *CCR5* HHG*2/HHA diplotype. His viral load was 744349 copies/mL and CD4⁺ T cell count was 4 cells/ml at 4 months prior to death. Both subjects were also lacking the *CCR2*-V64I variant and were heterozygous for *CCR5*-rs1799987/SNP #11 (Supplementary Table S2– S3).

4. DISCUSSION

The role of *CCR2* and *CCR5* in various chronic immunocompromised diseases and conditions, including atherosclerosis, asthma and HIV, has received growing attention. Importantly, they have been shown to have disparate allele frequencies and variable functional effects in Africans versus Europeans. Studies of *CCR2* and HIV-1 have focused primarily on the single non-synonymous variant V64I, with less attention to adjacent variants. We are aware of one other study that describes SNPs and haplotypes of *CCR2*, however only three of the *CCR2* SNPs described overlap with *CCR2* SNPs in this study [27].

In this study, sixteen SNPs in the *CCR2* and *CCR5* genes were genotyped. The data revealed six novel *CCR2* haplotype patterns and confirmed seven of the nine *CCR5* haplotypes described originally [16]. We used an ethnically diverse sample of subjects from the New York City area who self-identified as African American, European American and Hispanic American, and the allele frequencies in this sample resembled the frequencies observed in several public databases. At the time of the study, several of the SNPs investigated had limited allelic frequency information in the databases. In addition, few databases have information on Hispanics.

When we compared the LD plots of each ethnic group to the group as a whole, it was evident that the grouping masked unique ethnic-specific patterns. Separating the plots by ethnicity revealed LD in SNPs limited to Africans and Hispanics, and allowed us to observe the levels of variability between ethnic groups. As Africans represent phylogenetically older populations than Europeans, Africans are predicted to show patterns of increased variability compared to Europeans. Indeed, in our sample we found only four major *CCR2* haplotypes in Europeans, compared to six major haplotypes in Africans and Hispanics. In addition, the

African-specific alleles in the Hispanic sample are expected, considering that our cohort from New York City consists largely of individuals of Caribbean descent, who have significant ancestral mixture with Africans [28].

Because of the close proximity of the *CCR2* and *CCR5* genes, high LD can be found between SNPs in the two genes that may have functional importance. Several groups reported the impact of *CCR2*-V64I and *CCR5*-32 on delaying AIDS progression [6, 9]. We confirmed a link between *CCR2* SNP #4 (V64I) and the *CCR5* intronic SNP #15 (rs1800024), previously identified [7]. We also observed LD in Africans, between the synonymous *CCR2* SNP #6 (rs3918386, P339P) and the *CCR5* 5' UTR SNP #13 (rs41469351), not previously described in the literature. Allelic frequency data for SNP#13 was not reported in the public databases examined at the time of our study.

Interestingly, the *CCR2* haplotypes (H5-H6) that were limited to the African and Hispanic samples are comprised of SNPs that themselves are represented in all ethnicities, indicating that an ethnic-specific haplotype may be derived from a combination of SNPs that have variable ancestral origins. This finding also underlines the importance of studying haplotypes as opposed to single SNPs. Furthermore, while most studies were focused on the contribution of *CCR2*-V64I to HIV/AIDS progression, our data support the idea that adjacent *CCR2* SNPs and those in LD with *CCR2*-V64I such as the 3' UTR SNP #8 (rs34138562), and also the highly linked *CCR5* intronic SNP #15 (rs1800024), may be equally important genetic determinants that should be investigated. SNP #15 may prove to be an effective marker for *CCR2*-V64I. By focusing only on *CCR2*-V64I, earlier studies may have missed unique *CCR2* haplotypes that may have important function.

We present a rare case of homozygosity for the *CCR5*-32 deletion in a subject who was HIV infected and presented a high enough viral load to be clinically classified as an AIDS case. The subject did not have the *CCR2*-V64I variant. There are few cases in the literature of HIV-1 infected individuals who are homozygous for this deletion [29, 30]. It is speculated that in these rare instances, the HIV-1 virus may have infected a subject through an alternate co-receptor such as CXCR4 [11, 29, 30]. It is also possible that the infection was caused by some HIV strain or through some combination of unidentified molecules that support viral entry [11]. This provides further indication that the deletion is not completely protective.

In summary, this study demonstrates that variants/haplotypes in the genes encoding the chemokine receptors *CCR2* and *CCR5* show population-specific patterns that may contribute to disparities in disease infection or progression. Furthermore, it shows that in order to gain a wider scope of genetic loci that might contribute to pathogenesis in specific ethnicities, it is essential to study additional SNPs (and haplotypes) in specific ethnicities beyond the ones commonly studied so far.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. *CCR2* and *CCR5* genes show linkage in certain individuals and populations
2. Novel haplotypes for *CCR2* have been identified
3. The genotype of a rare case of an HIV + individual with the *CCR5* delta 32 deletion is discussed

Fig 1.

Genomic structure of the *CCR2* and *CCR5* genes. The location of genotyped SNPs are shown along the gene (see also Table 1).

Fig 2.

Haploview generated linkage disequilibrium (LD) plots of 8 *CCR2* SNPs #1–8) and 8 *CCR5* SNPs (#9–16) in the post mortem brain sample from three ethnicities (A–C). Numbers in each box represent r^2 value and black squares represent $r^2=1$. Squares go from dark to light to represent diminishing r^2 values.

Table 1

CCR2 and *CCR5* SNPs studied and minor allelic frequencies

Gene	SNP	Gene Location	Residue Change	Position build 37.3	Alleles	AF (n= 110)	EU (n=69)	HI (n=70)
<i>CCR2</i>	1 rs3918358	5' near gene		46394419	A>C	0.31 (0.29)	0.31 (0.33)	0.37 (0.41)
	2 rs3918359	5' near gene		46394680	T>A	0.05 (0.09)	0.22 (0.18)	0.13 (0.25)
	3 rs3092964	Exon 1 (5' UTR)		46395585	A>G	0.04 (0.09)	0.20 (0.19)	0.13 (0.25)
	4 rs1799864	Exon 2	Val64Ile	46399208	G>A	0.17 (0.18)	0.15 (0.11)	0.13 (0.22)
	5 rs1799865	Exon 2	Asn260Asn	46399798	T>C	0.31 (0.31)	0.33 (0.31)	0.37 (0.31)
	6 rs3918386	Exon 3	Pro339Pro	46401243	A>G	0.21 (0.19)	0.00 (0.00)	0.08 (0.04)
	7 rs743660	Exon 3 (3'UTR)		46402018	G>A	0.09 (0.06)	0.23 (0.23)	0.23 (0.21)
	8 rs34138562	Exon 3 (3'UTR)		46402053	T>G	0.22 (0.21)	0.15 (0.13)	0.14 (0.24)
<i>CCR5</i>								
	9 rs2856758	Exon 1 (5'UTR)		46411661	A>G	0.05	0.15	0.10
	10 rs2734648	Intron 1		46411840	G>T	0.38	0.45	0.31
	11 rs1799987	Intron 2		46411935	G>A	0.35	0.44	0.44
	12 rs1799988	Exon 2 (5'UTR)		46412259	T>C	0.45	0.48	0.42
	13 rs41469351	Exon 2 (5'UTR)		46412262	C>T	0.21	0.00	0.04
	14 rs1800023	Exon 2 (5'UTR)		46412308	A>G	0.11	0.37	0.33
	15 rs1800024	Intron 2		46412559	C>T	0.24	0.10	0.23
	16 rs333	Exon 3/ORF	frameshift	46414947	32	0.00	0.05	0.02

AF = African ancestry, EU = European ancestry, HI = Hispanic ancestry. Frequencies taken from public databases are in parentheses. ORF = open reading frame.

Table 2

CCR5 haplotypes and frequencies by ethnicity

SNPs		Haplotypes ^a										Frequencies		
9	10	11	12	13	14	15	16					AF ^b (n=12)	EU ^b (n=10)	HI ^b (n=16)
G	G	A	C	C	A	C	wt	HHG*1				0.13	0.05	0.03
G	G	A	C	C	A	C	32	HHG*2				0.00	0.15	0.03
A	G	A	C	C	A	T	wt	HHF				0.08	0.20	0.13
A	G	A	C	C	A	C	wt	HHE				0.17	0.15	0.16
A	T	G	T	T	A	C	wt	HHH				0.25	0.00	0.13
A	T	G	T	C	G	C	wt	HHG				0.13	0.40	0.34
A	G	G	T	C	A	C	wt	HHH				0.25	0.05	0.19

^a Haplotypes are labeled according to Gonzalez [16]

^b AF= African ancestry, EU= European ancestry, HI=Hispanic ancestry; Shaded area represents change from the presumed ancestral haplotype HHA

Table 3

CCR2 main haplotypes (>0.03), diplotypes and their frequencies by ethnicity

SNPs			Frequencies									
1	2	3	4	5	6	7	8	Haplotype	AF ^a (n=102)	EU ^a (n=64)	HI ^a (n=66)	
A	T	A	G	T	A	G	T	H1	0.28	0.51	0.42	
C	A	G	G	C	A	A	T	H2	0.07	0.22	0.21	
A	T	A	A	T	A	G	G	H3	0.15	0.14	0.13	
C	T	A	G	C	A	G	T	H4	0.20	0.08	0.14	
A	T	A	G	T	G	G	T	H5	0.19	0.00	0.08	
A	T	A	G	T	A	G	G	H6	0.05	0.00	0.00	
Diplotype												
AA	TT	AA	GG	TT	AA	GG	TT	H1/H1	0.04	0.13	0.11	
AC	AT	AG	GG	TC	AA	GA	TT	H1/H2	0.01	0.10	0.09	
AA	TT	AA	GA	TT	AA	GG	TG	H1/H3	0.03	0.08	0.04	
AC	TT	AA	GG	TC	AA	GG	TT	H1/H4	0.05	0.02	0.07	
AA	TT	AA	GG	TT	AG	GG	TT	H1/H5	0.06	0.00	0.02	
AC	AT	AG	GA	TC	AA	GA	TG	H2/H3	0.02	0.02	0.05	
CC	AT	AG	GG	CC	AA	GA	TT	H2/H4	0.01	0.02	0.05	
AC	TT	AA	GA	TC	AA	GG	TG	H3/H4	0.03	0.02	0.01	
AC	TT	AA	GG	TC	AG	GG	TT	H4/H5	0.06	0.00	0.01	
CC			GG	CC	AA	AA	TT	H2/H2	0.00	0.03	0.02	
AA	TT	AA	GA	TT	AG	GG	TG	H3/H5	0.03	0.00	0.01	
AC			GG	TC	AG	GA	TT	H2/H5	0.01	0.02	0.02	
AC	TT	AA	GG	TC	AA	GG	TG	H4/H6	0.01	0.00	0.01	
AA	TT	AA	AA	TT	AA	GG	GG	H3/H3	0.00	0.02	0.02	
AA			GA	TT	AA	GG	GG	H3/H6	0.02	0.00	0.00	
CC			GG	CC	AA	GG	TT	H4/H4	0.01	0.01	0.00	
AA	TT	AA	GG	TT	GG	GG	TT	H5/H5	0.00	0.00	0.01	
AA			GG	TT	AG	GG	TG	H5/H6	0.01	0.00	0.00	

^a AF= African ancestry, EU=European ancestry, HI=Hispanic ancestry; Shaded area represents change from the most frequent H1 haplotype or H1/H1 diplotype