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CR1 Knops blood group alleles are not associated with severe malaria in the Gambia

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The Knops blood group antigen erythrocyte polymorphisms have been associated with reduced falciparum malaria-based in vitro rosette formation (putative malaria virulence factor). Having previously identified single-nucleotide polymorphisms (SNPs) in the human complement receptor 1 (CR1/CD35) gene underlying the Knops antithetical antigens Sl1/Sl2 and McC^a/McC^b, we have now performed genotype comparisons to test associations between these two molecular variants and severe malaria in West African children living in the Gambia. While SNPs associated with Sl:2 and McC(b+) were equally distributed among malaria-infected children with severe malaria and control children not infected with malaria parasites, high allele frequencies for Sl 2 (0.800, 1365/1706) and McC^b (0.385, 658/1706) were observed. Further, when compared to the Sl 1/McC^a allele observed in all populations, the African Sl 2/McC^b allele appears to have evolved as a result of positive selection (modified Nei–Gojobori test Ka−Ks/s.e. = 1.77, P-value < 0.05). Given the role of CR1 in host defense, our findings suggest that Sl 2 and McC^b have arisen to confer a selective advantage against infectious disease that, in view of these case–control study data, was not solely Plasmodium falciparum malaria. Factors underlying the lack of association between Sl 2 and McC^b with severe malaria may involve variation in CR1 expression levels.
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Introduction

Rosette formation resulting from adhesion between *Plasmodium falciparum*-infected red blood cells (RBC) and uninfected RBC is viewed as a critical *in vitro* marker of malaria pathogenesis.[1] Parasitized RBCs (pRBC) adhere to uninfected RBC (rosetting), other pRBC (autoagglutination), and to endothelial cells lining blood vessels (cytoadherence). In aggregate, it is clear that these adhesive interactions underlie *P. falciparum* sequestration and are likely to contribute to the obstruction of blood flow and severe malaria. A number of field studies have reported an association between *in vitro* rosette formation using patient's cells collected at the time of clinical presentation of malaria illness and cerebral malaria,[1,2] severe malarial anemia,[3,4] or both cerebral malaria and severe malarial anemia.[5–8] Although exceptions to these findings have been reported,[9,10] these observations are of interest, as identifying host–parasite molecular partners underlying *P. falciparum* sequestration may provide direction for developing strategies to block interactions linked to severe malaria pathogenesis that contribute to millions of childhood deaths annually.

Serologic factors have been observed both to promote[6,11–14] and disrupt[1,2,5] *in vitro* rosette formation. Additionally, several recent studies have uncovered a complex of molecular relations involving the variant *P. falciparum* erythrocyte membrane protein (PfEMP1) family of pRBC surface antigens and a number of different host cell surface proteins in cellular adhesion assays. These studies have described 'receptor–ligand' relation between regions of PfEMP1 and CD36, thrombospondin (TSP), intercellular adhesion molecule-1 (ICAM-1),[15,16] complement receptor 1 (CR1/CD35),[17] platelet endothelial adhesion molecule-1 (PECAM-1/CD31),[18] blood group A,[19] and heparan sulfate-like molecules[20] in a range of cellular adhesion interactions. Of further interest has been the suggestion that these adhesive interactions may work synergistically to induce severe malaria.[4]

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Nomenclature for the Knops blood group Swain–Langley has been updated at the International Society of Blood Transfusion Nomenclature Committee meeting, 27th Congress of the ISBT, Vancouver, British Columbia as follows. The Swain–Langley-positive phenotype and antigen previously referred to as Sl(a+) and Sl^a are now Sl:1 and Sl1, respectively; the allele *Sl^a* is now *Sl 1*. The antithetical phenotype and antigen previously referred to as Vil+ and Vil are now Sl:2 and Sl2, respectively; the allele *Vil*, is now *Sl 2*. Nomenclature for the Knops blood group, McCoy phenotype, antigen, and allele are McC(a+), McC^a, *McC^a*, and McC(b+), McC^b, *McC^b* (Geoff Daniels, personal communication).

Our recent studies have suggested that low CR1 expression and the Sl:2 phenotype (previously known as Vil+[21]) are associated with reduced rosetting.[17] The Sl:2 phenotype may result from homozygosity for the *Sl2* allele, presence of a nonexpressed *Sl1* allele (amorph), and/or reduced expression levels of the *Sl1* allele that obscure serological typing assays. Through identification of single-nucleotide polymorphisms SNPs in exon 29[22] of the CR1 gene, we recently correlated specific amino-acid substitutions in complement control protein (CCP) module 25 (R1601G and K1590E) with Knops Sl:2 and McC(b+) serologic phenotypes, respectively. Here, we have performed genotype comparisons to test associations between these two molecular variants and severe malaria in a well-characterized case–control study of West African children living in the Gambia.[23]

Results

Consistent with previous studies,[22,24,25] we observed a significant elevation of the *Sl2* (1601G) and McC^b (1590E) alleles in the West African children (*Sl2*, 0.800, 1365/1706; McC^b, 0.386, 658/1706) compared to Caucasian (*Sl2*, 0.005, 1/200; McC^b, 0.000, 0/200), Asian (*Sl2*, 0.030, 6/198; McC^b, 0.020, 4/198), and Hispanic Americans (*Sl2*, 0.030, 6/200; McC^b, 0.025, 5/200) (*Sl2* Fisher's exact test, *P*-value <0.0001; McC^b Fisher's exact test, *P*-value <0.0001). Table 1 provides a comparison of genotype frequencies for these same populations. Overall, alleles at both the *Sl* and *McC* loci were observed to be in Hardy–Weinberg equilibrium (*Sl* χ^2 (2 df) $=0.79$, *P*-value $=0.674$; *McC* χ^2 (2 df) $=0.005$, *P*-value $=0.997$) for the Gambian children studied here. As the amino-acid substitution at position 1615 was not observed to influence serological recognition of either McCoy or Swain–Langley antigens,[22] and because all study subjects were either homozygous or heterozygous for the mutant allele (1615 V), this polymorphism was not considered in our association studies.

Results in Table 2 show that neither *Sl 2* nor McC^b alleles separately, or the *Sl* $2/McC^b$ allele (data not shown) conferred protection against any of the severe malaria phenotypes when malaria-infected children with disease were compared to children classified as 'non-malaria controls' (children with mild, mostly infectious, illnesses who did not require hospital admission and did not have any malaria parasites in their blood on microscopy).[23] Children classified by a generalized phenotype of severe malaria were further characterized by having one of cerebral malaria (unrousable coma score <3,[26] or repeated seizures of >30 min), severe anemia (hemoglobin <5 g/dl on admission), or by a combined category including death or disabling

Table 1 Comparing *Sl* (R1601G) and *McC* (K1590E) genotype frequencies: The Gambia *vs* North American ethnicities

Ethnic/geographic group	*n*	*R/R*[a]	*Freq.*	*R/G*	*Freq.*	*G/G*	*Freq.*	*P-value*[b]
Sl (R1601G)								
The Gambia	853	40	0.047	261	0.306	552	0.647	
Caucasian Am	100	99	0.990	1	0.010	0	0.000	<0.0001
Asian Am	99	94	0.949	4	0.040	1	0.010	<0.0001
Hispanic Am	100	94	0.940	6	0.060	0	0.000	<0.0001
McC (*K1590E*)	*n*	*K/K*	*Freq.*	*K/E*	*Freq.*	*E/E*	*Freq.*	*P-value*
The Gambia	853	324	0.380	400	0.469	129	0.151	
Caucasian Am	100	100	1.000	0	0.000	0	0.000	<0.0001
Asian Am	99	95	0.960	4	0.040	0	0.000	<0.0001
Hispanic Am	100	95	0.950	5	0.050	0	0.000	<0.0001

[a]Single letter amino-acid sequence nomenclature is used to identify CR1CCP25 alleles (R=arginine, G=glycine, K=lysine, E=glutamic acid).
[b]Fisher's exact test.

Table 2 Comparing *Sl* (R1601G) and *McC* (K1590E) genotype frequencies for Gambian mild control and severe malaria cohorts

Malaria phenotype	*n*	*R/R*[a]	*Freq.*	*R/G*	*Freq.*	*G/G*	*Freq.*	χ^2	*P-value*[b]
Sl (R1601G)									
Nonmalaria controls	390	23	0.059	128	0.328	239	0.613		
Malaria (severe)	463	17	0.037	133	0.287	313	0.676	4.7	0.095
Cerebral	331	13	0.039	95	0.287	223	0.674	3.4	0.182
Anemia	152	4	0.026	46	0.303	102	0.671	3.2	0.207
Death or sequelae	98	5	0.051	33	0.337	60	0.612	0.1	0.949
McC (*K1590E*)	*n*	*K/K*	*Freq.*	*K/E*	*Freq.*	*E/E*	*Freq.*	χ^2	*P-value*
Nonmalaria controls	390	155	0.397	171	0.438	64	0.164		
Malaria (severe)	463	169	0.365	229	0.495	65	0.140	2.8	0.247
Cerebral	331	124	0.375	160	0.483	47	0.142	1.6	0.450
Anemia	152	55	0.362	76	0.500	21	0.138	1.7	0.420
Death or Sequelae	98	40	0.408	49	0.500	9	0.092	3.4	0.181

[a]Single letter amino-acid sequence nomenclature is used to identify CR1CCP25 alleles (R=arginine, G=glycine, K=lysine, E=glutamic acid).
[b]Fisher's exact test.

neurological sequelae when discharged from the hospital.[27,28] For all comparisons, absence of association between the CR1CCP25 polymorphisms was independent of hemoglobin S in beta globin genotypes (the most significant human polymorphism conferring protection from severe malaria), and other potential confounders (sex, ethnic group, location of residence) (data not shown).

When DNA and amino-acid sequence comparisons were performed among human and *Pan troglodytes* CR1CCP25 haplotypes, we observed that all SNP led to amino-acid changes (nonsynonymous mutations) in the human alleles, whereas three of four SNP observed in the *P. troglodytes* CR1CCP25 coding sequence (GenBank Acc#. L24920)[29] did not change the amino-acid sequence (synonymous mutations). We then performed pairwise comparisons of the human CR1CCP25 sequences (Figure 1) to determine if evidence for positive or adaptive selection was observed. Results from a modified Nei–Gojobori test suggest that positive selection underlies the accumulation of sequence changes differentiating CR1CCP25*Hs*1 (*Sl1*/*McC*^{a}; all human ethnic/geographic populations) and CR1CCP25*Hs*5 (*Sl2*/*McC*^{b}; African-derived) (Modified Nei–Gojobori (Jukes–Cantor) test (*Ka-Ks*/s.e. = 0.023/0.013 = 1.77, *P*-value < 0.05).[30] Additional evidence supportive of positive selection among human CR1CCP25 haplotypes was also obtained by the McDonald–Kreitman test[31] (Fisher's exact test, two-tailed *P*-value = 0.03). Together with results from our previous study showing that the amino-acid substitutions at positions 1590 and 1601 are responsible for the serological phenotype differences between McC(a+) *vs* McC(b+) and Sl:1 *vs* Sl:2, respectively,[22] evidence consistent with positive selection suggests that the CR1CCP25 polymorphisms have evolved to confer some form of selective advantage in African populations.

Discussion

We observed a significant increase in the frequency of genetic polymorphisms associated with the Knops blood group phenotypes Sl:2 and McC(b+) in the Gambian study population living in malarious West Africa compared to non-African US-based populations. Despite the striking increased frequency of *Sl2* and *McC*^{b} in the Gambian study population, we did not observe an association between these African CR1 alleles and protection from severe malaria phenotypes.

To interpret this result, it may be important to consider the influence of erythrocyte CR1 expression polymorphism on the Knops serologic and malaria disease phenotypes. During our studies to identify DNA sequence polymorphisms associated with Sl:2 and McC(b+), we showed that both heterozygosity for the CR1 K1590E (SNP A4795G) and R1601G (SNP A4828G) amino-acid substitutions and erythrocyte CR1 expression polymorphism contributed to genotype-Knops blood group phenotype discordance in 18% of Malians studied.[22] With this in mind, we examined further the associations between *Sl* and *McC* genotypes and severe malaria by excluding heterozygous individuals from our analyses; however, again no significant associations between *Sl2*/*McC*^{b} alleles and severe malaria phenotypes were observed (data not shown).

Bellamy *et al*[32] have surveyed the same Gambian population studied here for a CR1 intron 27 *Hind* III polymorphism associated with CR1 expression level differences to determine if the low expression-associated allele would confer reduced susceptibility to severe malaria. Although no significant association was observed between the *Hind*III low expression-associated allele and protection from severe malaria,[32] it is important to consider the relationship between this polymorphism and CR1 expression in different ethnic groups. While a number of studies have observed an association between the *Hind*III low-expression-associated allele and reduced CR1 expression in Caucasians, Herrera *et al*[33] found no association between this marker and CR1 expression in African Americans. Similar lack of association between CR1 expression and the CR1 intron 27 *Hind* III polymorphism has now been observed in a study population from Mali.[34] Given the observed inconsistencies between CR1 genetic and expression phenotype polymorphisms, efforts to determine how erythrocyte CR1 expression differences contribute to *in vitro* rosetting and severe malaria would require assessing erythrocyte CR1 density using freshly collected blood samples not available for this retrospective study.

We have therefore confronted an interesting, but inevitable limitation of PCR-based field investigations. PCR has enabled innumerable field studies once constrained by difficulties in obtaining sufficient quantities and numbers of fresh blood samples, and by limited supplies of antisera needed for blood group serology. Adhesion between pRBC and uninfected cells may be influenced by amino-acid sequence polymorphism as well as differences in CR1 protein expression. Therefore, DNA-based studies may be limited in detecting associations between critical molecular polymorphisms and disease-related phenotypes.

It will also be important to keep in mind the potentially complex molecular interactions between the highly polymorphic *P. falciparum* ligand, PfEMP1, and CR1. The C3b binding site (recognized by the mAb J3B11) has recently been identified as the region of CR1 necessary for *P. falciparum* rosetting.[35] Interestingly, this region does not contain either *Sl2* or *McC*^{b} polymorphisms. Using a number of *P. falciparum* laboratory strains ($n = 4$) and field isolates from Kenya ($n = 15$) and Malawi ($n = 10$), it was shown that blocking interactions between erythrocyte-CR1 and *P. falciparum*-infected erythrocytes is consistently efficacious in inhibiting rosette formation and therefore not limited to one specific PfEMP1 sequence.[35] Thus, although initial characterization of CR1-PfEMP1 involvement with *in vitro* rosette formation relied upon a portion of a single PfEMP1 allele, this further study suggests that this host receptor–parasite ligand interaction may be a common biological mechanism responsible for adhesion between *P. falciparum*-infected and uninfected erythrocytes. Therefore, through an improved understanding of factors that might inhibit erythrocyte-CR1–*P. falciparum* interactions *in vitro*, it may be possible to develop therapeutic agents to limit or eliminate severe falciparum malaria pathogenesis.

Numerous microbial pathogens, in addition to *P. falciparum*, contribute to the infectious burden on African populations. The worldwide distribution of the *Sl1*/*McC*^{a} haplotype suggests that this apparent ancestral allele may have been carried by human populations

migrating out of Africa between 60 000 and 100 000 years ago. The observed restriction of the *Sl2/McC*^{b} haplotype within African populations suggests that this haplotype has emerged within African populations after this time period. Sequence comparisons between the human and chimpanzee CR1CCP25 domains, in general, and between the CR1 *Sl1/McC*^{a} (all human ethnic/geographic populations) and *Sl2/McC*^{b} (African-derived) domains suggest that this region of CR1 and resulting conformations[22] have evolved under adaptive selection pressure. This hypothesis could be tested more completely after more extensive DNA sequence analysis of the human CR1 locus using linkage analysis approaches recently described by Sabeti *et al*.[36] Since *Sl2/McC*^{b} appears to be advancing toward fixation in African populations (Table 1), and considering the important role CR1 plays in regulating immunity and host defense[37] this polymorphism has probably been elevated in frequency by infectious disease that, in view of these case–control study data, was not solely *P. falciparum* malaria.

Methods

Sample preparation

Samples analyzed here have been described previously.[23,38] Overall this was a hospital-based case–control study of children categorized according to malaria infection/disease severity as nonmalaria cases with other mild illnesses (nonmalaria controls; $n = 390$; average age = 2.47 years) or cases of severe malaria ($n = 463$; average age = 3.43 years).[23] Subgroups of severe malaria included cerebral malaria ($n = 331$ average age = 3.78 years), severe anemia ($n = 152$; average age = 2.19 years), and death or neurological sequelae ($n = 98$; average age = 3.53 years). Differences observed for the number of individuals studied here compared to previous studies arose based upon the availability of sufficient genomic DNA, efficiency of PCR and resolution of CR1 genotyping assay results. Samples representing North American ethnic groups have been described previously.[38] Briefly, anonymous donors were between 18 and 55 years of age and were classified by self-identified ethnicity. No information regarding exposure to malaria parasites or other infectious diseases was available. Sample collection from pediatric patients from the Gambia was performed following protocols approved by the Gambian government/MRC joint ethical committee and permission from each child's parent or guardian. Sample collection from random blood donors was performed following protocols approved by the American Red Cross.

CR1 genotyping using post-PCR multiplexed ligation detection reaction (LDR)

A 476 bp fragment of CR1 containing three polymorphic sites was PCR amplified using the primers 5′-TAAAAAATAAGCTGTTTTACCATACTC and 5′-CCCTCACACCCAGCAAAGTC (Figure 1). Amplifications were performed in a total volume of 25 µl and contained 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µM of each primer, approximately 50 ng DNA, and 0.5 U of Amplitaq Gold polymerase (Perkin-Elmer). To prevent polymerase extension during the subsequent LDR procedure, residual polymerase activity was removed by incubation with 1/10 volume of 1 mg/ml proteinase K in 50 mM EDTA at 37°C for 30 min, 55°C 10 min. The proteinase K was inactivated by incubation at 99°C for 10 min.

A set of three LDR probes consisting of two fluorescently labelled allelic and one common probe was designed for each of the SNPs occurring at nucleotide positions 4795, 4828, and 4870 (Table 3, Figure 2). The common probes (100 pmol of each) were 5′ phosphorylated

```
             1590       1600       1610       1620       1630       1640
CR1CCP25Hs1 STNKCTAPEV ENAIRVPGNR SFFSLTEIIR FRCQPGFVMV GSHTVQCQTN GRWGPKLPHC SR
CR1CCP25Hs2 ---------- ---------- ---T------ ---------- ---------- ---------- --
CR1CCP25Hs3 ---------- ---------- --------V- ---------- ---------- ---------- --
CR1CCP25Hs4 ---------- ----G----- --------V- ---------- ---------- ---------- --
CR1CCP25Hs5 ---E------ ----G----- --------V- ---------- ---------- ---------- --
```

Figure 1 Amino-acid sequence alignment comparing CR1CCP25 (*Hs*) alleles. Standard single-letter nomenclature illustrates the amino-acid sequence for five human CR1CCP25 alleles. This sequence is inclusive of amino acids 1587–1648. Amino-acid identity is represented by a dash (-); polymorphic positions are identified by a variant amino-acid symbol compared to the CR1CCP25*Hs*1 reference sequence. GenBank accession numbers for each of the human CR1CCP25 alleles include AF264716 (CRlCCP25*Hs*1), AF264715 (CR1CCP25*Hs*2), L17408 (CR1CCP25*Hs*3), AF169969 (CR1CCP25*Hs*4), and AF169970 (CR1CCP25*Hs*5).

Table 3 CR1 exon 29[a]-specific ligase detection reaction genotyping assay probes

LDR probes	*LDR probe sequence*	*Length (bp)*
4795A	FAM-aataCAGCCCTCCCCCTCGGTGTATTTCTACTAATA	71
4795G	HEX-ataaAGCCCTCCCCCTCGGTGTATTTCTACTAATG	70
4795com	AATGCACAGCTCCAGAAGTTGAAAATGCAATatat	
4828Crev	HEX-GAGGGAAAAGAAACTCCTGTTTCCTGGTACTCC	65
4828Trev	FAM-GTGAGGGAAAAGAAACTCCTGTTTCCTGGTACTCT	67
4828revcom	AATTGCATTTTCAACTTCTGGAGCTGTGCATT	
4870G	FAM-AAACAGGAGTTTCTTTTCCCTCACTGAGATCG	61
4870A	HEX-GAAACAGGAGTTTCTTTTCCCTCACTGAGATCA	62
4870com	TCAGATTTAGATGTCAGCCCGGGTTTGTC	

[a]Exon 29 encodes CR1CCPs 24 and 25.

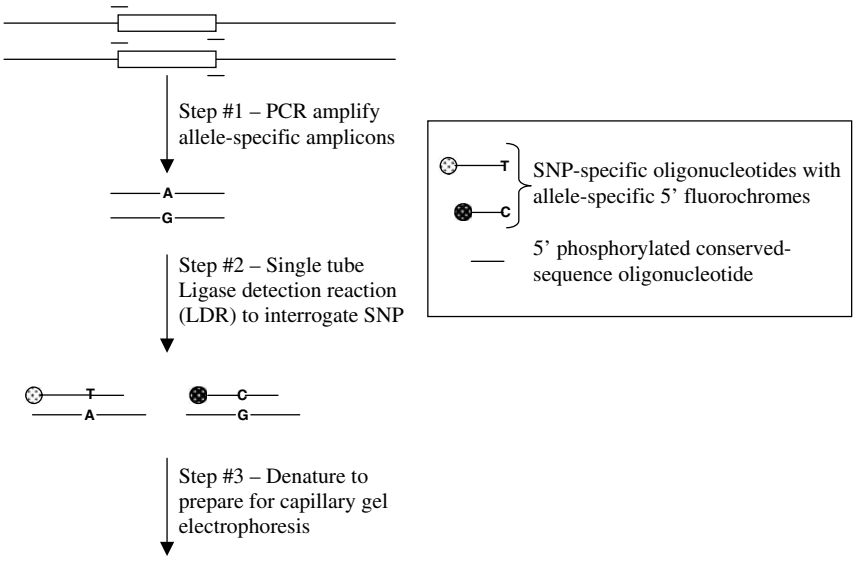

Figure 2 Post-PCR ligase detection reaction (LDR) genotyping assay.

in a volume of 100 µl using 10 U of T4 polynucleotide kinase and 1 mM ATP in 70 mM Tris-HCl buffer pH 7.6 containing 10 mM $MgCl_2$ and 5 mM dithiothreitol. After incubation at 37°C for 45 min, the T4 kinase was inactivated by the addition of 20 µg of proteinase K in 100 µl of TE buffer (10 mM Tris/HCl and 5 mM EDTA pH 8.0) and incubated at 37°C for 30 min. The proteinase K was inactivated by incubation at 99°C for 10 min.

Multiplexed LDR was performed in 15 µl volumes of 20 mM Tris-HCl buffer pH 7.6 containing 25 mM KCl, 10 mM $MgCl_2$, 1 mM NAD^+, 10 mM DTT, 0.1% Triton X-100, 10 nM (200 fmol) of each LDR probe, 2 µl of PCR product, and 2 U of *Taq* DNA ligase. The reactions were prepared on ice and then hot-started by placing on PCR blocks preheated to 95°C. An initial 1 min denaturation at 95°C was followed by 15 thermal cycles of denaturation at 95°C for 15 s, annealing and ligation at 68°C for 4 min. The activity of the ligase was stopped after 15 cycles by cooling to 4°C and adding 2 µl of 100 mM EDTA.

Electrophoresis and detection of LDR products was performed on an ABI 3700 fluorescent DNA sequencer. To 1 µl of LDR product was added 10 µl Hi Di formamide and 0.01 µl of 8 nM ABI Genescan-500 Rox size standards. Product sizes were calculated relative to the standard by Genescan 3.5 using the second-order least-squares method. Automated allele calling of LDR products was performed using Genotyper 2.5 and standardized by assays performed on amplicons from cloned and sequenced allele-specific controls.

Hemoglobin (Hb) A/S genotyping

For individual samples lacking analysis of Hb A/S status, genotyping was performed following methods developed by Husain *et al*.[39] In these analyses, numerous samples examined previously were included.[23] All samples assayed in this, and the former study produced results that were 100% concordant (data not shown).

Statistical analysis

χ^2 and Fisher's exact tests were performed using Stat View 5.0.1 or SAS 8.02 (SAS Institute, Inc., Cary, NC, USA). Tests comparing nonsynonymous (amino-acid changing; *Ka*) SNPs *vs* synonymous (amino-acid non-changing; *Ks*) between allele-specific sequences were performed using MEGA2 (http://www.megasoftware.net).[30] Briefly, modified Nei–Gojobori (Jukes–Cantor) tests were performed for all pairwise sequence comparisons among the human haplotypes reported in Figure 1 to determine the difference ($Ka-Ks$); standard errors (s.e.) were computed based upon 1000 bootstrap replicates. A further one-tailed Z-test (D/σ) was performed to determine the significance of each pairwise difference: $Ka-Ks$/(s.e.) where a score $\geqslant 1.6$ attains a *P*-value of <0.05. An additional test of positive/adaptive selection was conducted by performing a McDonald–Kreitman test comparing the five human CR1CCP25 haplotypes with that for *P. troglodytes* (GenBank Acc# L24920).[29] This test was performed using DnaSP 3.53 (http://www.ub.es/dnasp).[40]

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