Polymorphisms of CUL5 Are Associated with CD4+ T Cell Loss in HIV-1 Infected Individuals

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Human apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3 (ApoB3c) antiretroviral factors cause hypermutation of proviral DNA leading to degradation or replication-incompetent HIV-1. However, HIV-1 viral infectivity factor (Vif) suppresses ApoB3c activity through the Cullin 5-Elongin C E3 ubiquitin ligase complex. We examined the effect of genetic polymorphisms in the CUL5 gene (encoding Cullin 5 protein) on AIDS disease progression in five HIV-1 longitudinal cohorts. A total of 12 single nucleotide polymorphisms (SNPs) spanning 93 kb in the CUL5 locus were genotyped and their haplotypes inferred. A phylogenetic network analysis revealed that CUL5 haplotypes were grouped into two clusters of evolutionarily related haplotypes. Cox survival analysis and mixed effects models were used to assess time to AIDS outcomes and CD4+ T cell trajectories, respectively. Relative to cluster I haplotypes, the collective cluster II haplotypes were associated with more rapid CD4+ T cell loss (relative hazards [RH] = 1.47 and p = 0.009), in a dose-dependent fashion. This effect was mainly attributable to a single cluster II haplotype (Hap10) (RH = 2.49 and p = 0.00001), possibly due to differential nuclear protein–binding efficiencies of a Hap10-specifying SNP as indicated by a gel shift assay. Consistent effects were observed for CD4+ T cell counts and HIV-1 viral load trajectories over time. The findings of both functional and genetic epidemiologic consequences of CUL5 polymorphism on CD4+ T cell and HIV-1 levels point to a role for Cullin 5 in HIV-1 pathogenesis and suggest interference with the Vif-Cullin 5 pathway as a possible anti-HIV-1 therapeutic strategy.

Introduction

Members of the apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3 (ApoB3c) family of cytidine deaminases are innate cellular anti-HIV-1 factors [1,2]. In the absence of HIV-1 viral infectivity factor (Vif), both ApoB3cG and ApoB3cF are packaged into HIV-1 virions and during reverse transcription in the newly infected cell deaminate dC to dU in the nascent minus-strand DNA. This deamination results in either the degradation of the cDNA through a cellular uracil-DNA-glycosidase degradation pathway or pervasive G to A hypermutation in the plus-strand proviral cDNA [3–7]. However, the antiretroviral activities of ApoB3cG and ApoB3cF are suppressed by HIV-1 Vif, effectively preventing incorporation of ApoB3cG or ApoB3cF into virions, primarily by inducing ApoB3cG degradation by proteasomes [8–11], and perhaps by additional mechanisms [5,12,13]. HIV-1 Vif interacts with the cellular proteins Cullin 5, Elongin B, Elongin C, and Rbx1 to form an E3 ubiquitin ligase complex that induces polyubiquitination and proteasomal degradation [7]. When the Cullin 5 complex is inhibited by mutating Cullin 5 or is down-regulated by RNA interference, Vif-induced polyubiquitination and degradation of ApoB3cG is blocked [7,14]. This suggests that the ability of HIV-1 Vif to suppress the antiviral activity of the two ApoB3c proteins specifically depends on Cullin 5-Elongin B-Elongin C function [7,14]. Most recently, the Vif-Cullin 5 binding domain has been mapped to a highly conserved HCCH motif within the HIV-1 Vif zinc-binding domain [15,16]. The region in Cullin 5 that mediates Vif interaction has been mapped to the loop region between helices 6 and 7 (amino acids 120–138) [16]. In an independent report, the Vif interaction region was...

Abbreviations: AA, African Americans; adj, adjusting; AIC, Akaike Information Criteria; ALIVE, AIDS Link to the Intravenous Experience; ApoB3c, apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3; ct, cluster tagging; EA, European Americans; EMSA, electrophoretic mobility-shift assay; FDR, false discovery rate; HC, Han Chinese; HIV-1, HIV type 1; ht, haplotype tagging; IL, interleukin; LD, linkage disequilibrium; MACS, Multicenter AIDS Cohort Study; MHCS, Multicenter Hemophilia Cohort Study; RH, relative hazards; SC, seroconverters; SN, seronegatives; SNP, single nucleotide polymorphism; Vif, viral infectivity factor

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Author Summary

Human apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3 G (Apobec3G) is an innate antiviral protein that inhibits HIV type 1 (HIV-1) replication by causing deleterious mutations in the HIV-1 genome. Unfortunately, HIV-1 has a strategy to defeat the antiviral activity of Apobec3G. The HIV-1 viral infectivity factor (Vif) binds to Apobec3G leading to the degradation of Apobec3G through a complex containing Cullin 5 and the proteins Elongin B and Elongin C. Since Cullin 5 directly interacts with Vif and is critical to the Apobec3G degradation pathway, the authors asked if genetic variation of CUL5 could tip the balance between HIV-1 and Apobec3G and modify the course of HIV-1 infection. They showed that genetic variation in the CUL5 gene encoding Cullin 5 affected the rate of CD4+ T cell loss in patients infected with HIV-1. CUL5 haplotypes formed two clusters of evolutionarily related haplotypes with opposing effects—cluster I delayed and the cluster II accelerated CD4+ T cell loss. The effect was mainly attributable to a single haplotype or its tagging-SNP, which demonstrated differential binding of transcription factors. This finding highlights the epidemiologic importance of the HIV-1 and Cullin 5 interaction and suggests that the factors in the HIV-1 Vif-Apobec3G degradation pathway may be targets for antiviral drugs.

Results

Description of CUL5 Variants

The CUL5 gene is approximately 100 kb in length and consists of 19 exons (Figure 1). At the start of this project, about 40 SNPs for the CUL5 gene on human Chromosome 11q22 had been deposited in the National Center for Biotechnology Information database SNP (NCBI dbSNP) (http://www.ncbi.nlm.nih.gov/SNP/). No nonsynonymous nucleotide replacements have been reported to date. Through resequencing of 188 DNA samples, we did not discover any additional SNPs in exons 15–19 in the C terminus of the CUL5 gene or in the putative promoter region. By considering SNP location, spacing, and allele frequency, 12 SNPs were selected for genotyping in the AIDS cohorts (Figure 1 and Table S1). Of these 12 SNPs, one SNP (SNP5, rs7117111) is within the coding region, one (SNP12) is in the 3′ UTR, and the remaining SNPs are in introns. SNP5 is a synonymous transition (CAA > CAG) at the third position of codon 75 encoding glutamine (Q). Each of the 12 SNPs was in Hardy-Weinberg equilibrium (p > 0.05) in African Americans (AA) and European Americans (EA).

CUL5 SNPs Exhibit Strong Linkage Disequilibrium

The 12 SNPs span 93 kb of the CUL5 gene (Figure 2A and 2B). The extent of linkage disequilibrium (LD) was assessed by calculating all pairwise D′ values among the CUL5 SNPs, separately for AA and EA (Figure 2A and 2B). Strong LD was observed among all SNPs (D′ range 0.92–1.0) in EA and almost all SNPs in AA, with the majority of marker pairs showing D′ values between 0.95 and 1.0. In both AA and EA, as well as in a Han Chinese (HC) population, SNPs 3, 5, and 8 and SNPs 7 and 9 were in perfect LD (D′ = 1 and r2 = 1). Thus, each SNP carries the same information content as its proxies. We therefore used SNP5 as a proxy for SNPs 3 and 8 and SNP7 as a proxy for SNP9. SNP5 has a diverse allele frequency assessed therein has no obvious effect on susceptibility to HIV-1 infection.

Effect of CUL5 SNPs and Haplotypes on Susceptibility to HIV-1 Infection

We compared the CUL5 allele and haplotype frequency distributions among highly exposed but uninfected individuals to HIV-1 seronegative individuals (SN) and HIV-1-infected seroconverter individuals (SC). No distortion of frequency distribution between risk groups was observed for any SNP or haplotype in AA or EA (unpublished data), suggesting that the CUL5 genetic variation assessed herein has no obvious effect on susceptibility to HIV-1 infection.

CUL5 Clusters Were Associated with CD4+ T Cell Depletion in African Americans

AA and EA were analyzed separately since the allele frequencies and haplotype structures differed between the two groups. To minimize the haplotypes and SNPs to be

mapped to the first cullin repeat (amino acids 1–158) of Cullin 5 [17].

We recently reported that a nonsynonymous single nucleotide polymorphism (SNP) in the APOBEC3G gene may be associated with altered AIDS progression [18]. Since Cullin 5 is a critical host factor in the Vif-mediated degradation pathway of anti-HIV-1 proteins Apobec3G and Cullin 5 is a critical host factor in the Vif-mediated degradation pathway of anti-HIV-1 proteins Apobec3G and modify the course of HIV-1 infection. They showed that genetic variation in the CUL5 gene encoding Cullin 5 affected the rate of CD4+ T cell loss in patients infected with HIV-1. CUL5 haplotypes formed two clusters of evolutionarily related haplotypes with opposing effects—cluster I delayed and the cluster II accelerated CD4+ T cell loss. The effect was mainly attributable to a single haplotype or its tagging-SNP, which demonstrated differential binding of transcription factors. This finding highlights the epidemiologic importance of the HIV-1 and Cullin 5 interaction and suggests that the factors in the HIV-1 Vif-Apobec3G degradation pathway may be targets for antiviral drugs.
Figure 1. Gene Map, SNPs, and Haplotypes in the Human CUL5 Gene

Coding exons are marked by black blocks, and 5' and 3' UTR by white blocks. Nucleotide changes and frequencies of SNPs and haplotypes (Hap) in AA, EA, and HC are presented. ctSNPs are shown in color.

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Figure 2. LD of CUL5 SNPs in AA and EA

LD of CUL5 SNPs is shown in AA (A) and EA (B). Pairwise D' plots were generated using Haploview with its standard color scheme. Dark-red squares indicate high D' values, light-blue squares indicate high D' values with low LOD scores, and light-red and white squares indicate low D' values. D' values were indicated for those not equal to 1.0. A single LD block was defined for both AA and EA under the default confidence interval criteria. A reduced-medium network for the genealogical relationship of CUL5 haplotypes is shown in AA (C) and EA (D). The network was inferred in terms of mutational distance, on the basis of 12 CUL5 SNPs and one chimpanzee (Chimp) sequence. Median vector (mv1), the consensus sequences inferred by parsimony criteria, represents possible unsampled sequences or extinct ancestral sequences. Haplotypes (H1–H11) are represented by circles, whose area reflects the number of alleles observed in each population. The solid branches between haplotypes represent mutational events or SNPs (S1–S12). The circles in green show haplotypes with detrimental effect and those in blue show protective effect on AIDS progression in the Cox model analysis; the protective effect of H3 in light blue was of less certainty (see Results). The haplotypes were separated into two clusters, cluster I and II, carrying ctSNPs A or G, respectively. Cluster I and II in AA are shaded in blue and green, respectively. SNP2 is omitted in (B) and (D) as it was absent in EA.

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haplotypes) on progression to CD4⁺ T cells. Cox model analysis, the G allele significantly influenced the risk of dropping to CD4⁺ T cells, and that this effect is dose-dependent: the three GG homozygous individuals developed T cell depletion (RHadj = 2.49 and p = 0.003, log-rank) (Figure 3B). Because Hap10 carries both the htSNP6 G allele and the ctSNP5 G allele, we tested ctSNP5 adjusting for the effects of htSNP6 within a Cox model. The ctSNP5 effect became nonsignificant (RHadj = 1.25 and p = 0.170), suggesting that most of the association of ctSNP5 G allele and other cluster II haplotypes with CD4⁺ T cell loss was due to htSNP6. When considering ctSNP5 as a confounding covariate, the association remained robust for htSNP6 (RHadj = 2.16 and p = 0.0009). However, not all of the ctSNP5 association with CD4⁺ T cell decline could be attributed to htSNP6. This can be readily observed in Figure 3C where individuals homozygous for cluster I haplotypes (ctSNP5 A/A) are protective relative to cluster II carriers, and the greatest risk is for carriers of the cluster II Hap10 (p = 0.0002, log-rank). This suggests that one or more ctSNP5 G-bearing haplotypes and the htSNP6 G-bearing Hap10 may be tracking the same functional allele elsewhere in the CUL5 gene.

Hap1 and Hap6 in cluster I also were associated with delayed time to CD4⁺ < 200 (RH = 0.59, 0.41; p = 0.013, 0.02, respectively) (Table 2 and Table S2). None of the SNPs or haplotypes was significantly associated with progression to the AIDS late-stage endpoint AIDS-87 (Table 1 and Table S2).

### Relative Contribution of CUL5 Haplotypes on CD4⁺ T Cell Depletion

To evaluate the relative contributions of the protective Hap1 and Hap6 haplotypes and the accelerating Hap10 on time to CD4⁺ < 200, the Akaike Information Criteria (AIC) were used to select the best Cox proportional hazards model. The covariates HLA homozygosity and HLA B57 were used in the base mode (model 1). The smallest AIC was achieved for

| SNP Model | Endpoint | Genotype | n | RH 95% CI | p | q* | RH 95% CI | p | q |
|-----------|----------|----------|---|-----------|---|----|-----------|---|----|
| ctSNP5‡  | Reference | AA       | 108 | 1  | — — — 1 — — — | 2  | — — — | 1 — — — | 2  | — — — |
| ctSNP5‡  | Heterozygotes | CD4 < 200 | AG | 141 | 1.35 | 0.86, 2.11 | 0.20 | — — — | 1.27 | 0.80, 2.01 | 0.31 | — — — |
| ctSNP5‡  | Homozygotes | CD4 < 200 | GG | 41  | 2.52 | 1.42, 4.46 | 0.002 | 0.012 2.34 | 1.28, 4.27 | 0.006 | 0.025 |
| ctSNP5‡  | Additive  | CD4 < 200 | GG > AG > AA | 290 | 1.56 | 1.17, 2.07 | 0.002 | 0.013 1.47 | 1.10, 1.96 | 0.009 | 0.026 |
| Additive, adjusted SNPs§ | CD4 < 200 | GG > AG > AA | 290 | 1.37 | 1.01, 1.87 | 0.04 | 0.068 1.25 | 0.91, 1.71 | 0.17 | 0.195 |
| Additive | AIDS-93  | GG > AG > AA | 291 | 1.36 | 1.05, 1.76 | 0.02 | 0.043 1.33 | 1.04, 1.71 | 0.026 | 0.050 |
| Additive | AIDS-87  | GG > AG > AA | 293 | 1.10 | 0.77, 1.58 | 0.6 | — — | 1.07 | 0.75, 1.51 | 0.71 | — — |
| Additive, adjusted SNPs§ | Reference | AA | 260 | 1  | — — — 1 — — — | 1  | — — — | 1 — — — | 1  | — — — |
| Additive | AIDS-93  | GG > AG > AA | 291 | 2.06 | 1.32, 3.20 | 0.001 | 0.008 2.11 | 1.40, 3.19 | 0.004 | 0.007 |
| Additive | AIDS-87  | GG > AG > AA | 293 | 0.99 | 0.44, 2.22 | 0.98 | — — | 1.27 | 0.63, 2.56 | 0.51 | — — |

*The q-value (q) measures the FDR and was presented for those with p < 0.05.

‡Adjusted for HLA homozygosites and B57.

§SNP5 is a proxy for SNP3 and SNP8 and also for the haplotype cluster (ct).

Additional adjusted for SNP6.

*Additionally adjusted for SNP5.

CI, confidence interval; RH, relative hazards

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tested, we took advantage of the unique haplotype relationship in CUL5 revealed in the haplotype network. We first tested the hypothesis that the two major clusters of haplotypes were differentially associated with disease progression in AA and EA. Cluster II identified by the ctSNP5 G allele was significantly associated with accelerated rates of progression to CD4⁺ < 200 in both the unadjusted analysis and after adjusting (adj) for the confounding effects of HLA B57 and HLA homozygosity in the Cox proportional hazards model in AA (Table 1). When an additive genetic effect was tested in the Cox model analysis, the G allele significantly influenced the risk of dropping to CD4⁺ < 200 (relative hazards [RH]adj = 1.47 and p = 0.009). No significant associations were found for AIDS-1987 (Table 1). Kaplan-Meier survival curves stratified for the ctSNP5 AA, AG, and GG genotypes suggested an additive effect of the G allele (and by inference cluster II haplotypes) on progression to CD4⁺ < 200 (p = 0.003, log-rank) (Figure 3A). These results suggest that cluster II haplotypes, all of which carry ctSNP5 G, were associated with more rapid loss of CD4⁺ T cells, and that this effect is dose-dependent: individuals bearing one or two haplotypes from cluster II are at greater risk relative to individuals bearing any two haplotypes from cluster 1 (Figure 3A).

### CUL5 Cluster I and II Contain Protective and Detrimental Haplotypes, Respectively

We next examined the role of each haplotype to identify specific high-risk haplotypes. The Cox model analysis indicated that the haplotype-tagging (ht)SNP6 G allele carried only on Hap10 (f = 5%) was a strong risk factor for CD4⁺ T cell depletion (RHadj = 2.49 and p = 0.00001, for the additive model) (Figure 3B and Table 1). In a Kaplan-Meier survival plot, the htSNP6 G allele was a risk factor for CD4⁺ < 200: the three GG homozygous individuals developed CD4⁺ < 200 within five years (and all died within six years), and all AG heterozygotes developed CD4⁺ < 200 within nine years of seroconversion (p = 0.0003, log-rank and p = 0.008, Wilcoxon) (Figure 3B).

To evaluate the relative contributions of the protective Hap1 and Hap6 haplotypes and the accelerating Hap10 on time to CD4⁺ < 200, the Akaike Information Criteria (AIC) were used to select the best Cox proportional hazards model. The covariates HLA homozygosity and HLA B57 were used in the base mode (model 1). The smallest AIC was achieved for...
model 5 where all three haplotypes were added in model 5 (Table S3).

Role of CUL5 SNPs and Haplotypes on AIDS-Free Survival in European Americans

The six haplotypes in EA also form two clusters (Figure 2D). In the Cox model analysis, ctSNP5 showed no significant associations (Table S4), although weak associations were observed for two haplotypes and three SNPs. The cluster I haplotype Hap3 had an additive protective effect on rate of progression to CD4⁺, 200 and AIDS 1993 (RH = 0.71–0.68 per allele, p = 0.026–0.006, respectively), and the cluster I Hap7 had a slight accelerating effect (CD4⁺, 200, RH = 1.25, p = 0.056; AIDS-93, RH = 1.31, p = 0.010, respectively) (Table S2). Small effects were also observed for SNP4 (protective for AIDS-93, RH = 0.78, p = 0.03), SNP7 (risk for AIDS-93, RH = 1.24, p = 0.02), and SNP12 (risk for AIDS-93, RH = 1.2, p = 0.02) (Table S4).

Effect of CUL5 SNPs and Haplotypes on Longitudinal CD4⁺ T Cell Counts and HIV-1 RNA Levels in African Americans from the AIDS Link to the Intravenous Experience Cohort

CD4⁺ T cell counts and HIV-1 RNA levels were measured multiple times during the follow-up period for the AIDS Link to the Intravenous Experience (ALIVE) SC participants. An average of 7.8 and 6.8 measurements of CD4⁺ T cell counts and viral load, respectively, per patient for up to nine years from the seroconversion to 1997-censoring date were available for analysis. We evaluated the effects of ctSNP5 (representing all cluster II haplotypes) and htSNP6 (Hap10) on the longitudinal slope of CD4⁺ T cell count and HIV-1 RNA level over the clinical course using the linear mixed random effects model.

The ctSNP5 G allele tends to be associated with gradient differences for CD4⁺ T cell slopes stratified by genotype (Figure 4 and Table 3): each copy of the ctSNP5 G allele was associated with /C0 0.99 (p = 0.05) or /C0 1.23 (p = 0.01) lower mean CD4⁺ T cell trajectory, over the observation periods from seroconversion to July 31, 1997 (date censored before highly active antiretroviral therapy [HAART]) or to July 31, 2004, respectively (Table 3). The htSNP6 G allele on Hap10 was strongly associated with a more rapid mean loss of CD4⁺ T cells: each copy of the ctSNP6 G allele was associated with /C0 2.56 (p = 0.01) or /C0 2.63 (p = 0.01) lower mean CD4⁺ T cell trajectory, for the observation periods from seroconversion to July 31, 1997 or to July 31, 2004, respectively (Table 3).

Effect of CUL5 SNPs and Haplotypes on Longitudinal CD4⁺ T Cell Counts and HIV-1 RNA Levels in African Americans from the AIDS Link to the Intravenous Experience Cohort

HIV-1 RNA level over the clinical course using the linear mixed random effects model.

The results of Cox Model Analysis were from the additive model. Results shown were unadjusted and those with adjustment were similar. Events are for the number of individuals who experienced the endpoint. CI, confidence interval; n, number of individuals; p, p-values; q, q-values (measures for the FDR); RH, relative hazards.

Table 2. Effects of Selected CUL5 Haplotypes on AIDS Progression in AA by Cox Model Analysis

| Haplotype | Endpoint    | n | Events | RH   | 95% CI  | p     | q  |
|-----------|-------------|---|--------|------|---------|-------|----|
| Hap1      | CD4 < 200   | 239 | 88     | 0.59 | 0.39, 0.90 | 0.013 | 0.031 |
| Hap1      | AIDS-87    | 242 | 49     | 0.56 | 0.31, 1.01 | 0.054 |
| Hap6      | CD4 < 200   | 239 | 88     | 0.47 | 0.23, 0.97 | 0.042 | 0.068 |
| Hap6      | AIDS-87    | 242 | 49     | 1.08 | 0.51, 2.31 | 0.832 |
| Hap7      | CD4 < 200   | 239 | 88     | 1.28 | 0.61, 2.69 | 0.507 |
| Hap7      | AIDS-87    | 242 | 49     | 2.66 | 1.26, 5.63 | 0.010 | 0.026 |
| Hap10     | CD4 < 200   | 239 | 88     | 2.57 | 1.49, 4.41 | 0.001 | 0.007 |
| Hap10     | AIDS-87    | 242 | 49     | 1.54 | 0.66, 3.59 | 0.319 |

The results of Cox Model Analysis were from the additive model. Results shown were unadjusted and those with adjustment were similar. Events are for the number of individuals who experienced the endpoint. CI, confidence interval; n, number of individuals; p, p-values; q, q-values (measures for the FDR); RH, relative hazards.

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Figure 3. Kaplan-Meier Survival Analysis of CUL5 Variants on Progression from Seroconversion to CD4⁺ T cells < 200 /mm³ in AA
(A) Shows ctSNP5 (the cluster); (B) shows htSNP6 (Hap10); and (C) shows compound genotypes of ctSNP5 and htSNP6.
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Table 2. Effects of Selected CUL5 Haplotypes on AIDS Progression in AA by Cox Model Analysis

| Haplotype | Endpoint    | n | Events | RH   | 95% CI  | p     | q  |
|-----------|-------------|---|--------|------|---------|-------|----|
| Hap1      | CD4 < 200   | 239 | 88     | 0.59 | 0.39, 0.90 | 0.013 | 0.031 |
| Hap1      | AIDS-87    | 242 | 49     | 0.56 | 0.31, 1.01 | 0.054 |
| Hap6      | CD4 < 200   | 239 | 88     | 0.47 | 0.23, 0.97 | 0.042 | 0.068 |
| Hap6      | AIDS-87    | 242 | 49     | 1.08 | 0.51, 2.31 | 0.832 |
| Hap7      | CD4 < 200   | 239 | 88     | 1.28 | 0.61, 2.69 | 0.507 |
| Hap7      | AIDS-87    | 242 | 49     | 2.66 | 1.26, 5.63 | 0.010 | 0.026 |
| Hap10     | CD4 < 200   | 239 | 88     | 2.57 | 1.49, 4.41 | 0.001 | 0.007 |
| Hap10     | AIDS-87    | 242 | 49     | 1.54 | 0.66, 3.59 | 0.319 |

The results of Cox Model Analysis were from the additive model. Results shown were unadjusted and those with adjustment were similar. Events are for the number of individuals who experienced the endpoint. CI, confidence interval; n, number of individuals; p, p-values; q, q-values (measures for the FDR); RH, relative hazards.

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Figure 3. Kaplan-Meier Survival Analysis of CUL5 Variants on Progression from Seroconversion to CD4⁺ T cells < 200 /mm³ in AA
(A) Shows ctSNP5 (the cluster); (B) shows htSNP6 (Hap10); and (C) shows compound genotypes of ctSNP5 and htSNP6.
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was obtained using the extended followed-up time to July 31, 1997. CD4 association (respectively), in the data censored in July 31, 1997. A stronger alleles on the mean CD4 group, we tested the effects of different combinations of APOBEC3G (Tables S2–S4).

The observation period was from seroconversion to the censoring date of July 31, 1997. CD4 T cell counts were measured at 6-month intervals and were square-root transformed with standard error represented by the vertical bar. doi:10.1371/journal.pgen.0030019.g004

Integrations between APOBEC3G-H186R and CUL5 SNPs

Because Cullin 5 affects Apobec3G’s anti-HIV-1 activity, we reasoned that the CUL5 cluster II haplotypes may interact with the APOBEC3G-186R allele, previously reported by us to be associated with accelerated CD4 T cell depletion [18]. Using the group of individuals homozygous for both APOBEC3G-186H and cluster I haplotypes as a reference group, we tested the effects of different combinations of alleles on the mean CD4 T cell trajectories using the mixed effects model (Table 3). We observed a significant additive interaction between APOBEC3G-186R carriers and ctSNP5 G or htSNP6 G carriers (−2.88, p = 0.01 and −3.64, p = 0.02, respectively), in the data censored in July 31, 1997. A stronger association (−3.11, p = 0.005 and −4.16, p = 0.007, respectively) was obtained using the extended followed-up time to July 31, 2004 (Table 3).

Estimate of the False Discovery Rates

To account for the multiple comparisons made in this study, we estimated the q-value statistic to estimate the false discovery rate (FDR). The FDR was calculated incorporating all p-values from 122 tests performed for SNPs and haplotypes in the Cox model and mixed effects model. The q-value was obtained for each and all of the p-values, and the q-values for those with p ≤ 0.05 were presented. The associations for ctSNP5, htSNP6 with CD4 < 200, and CD4 T cell slope in AA as well as Hap1, Hap7 in AA, and Hap3 in EA had q-values below the stringent cutoff of 0.05 indicating a FDR of 5% for a given p-value (Tables 1–3 and Tables S2–S4).

Electrophoretic Mobility-Shift Assay

To explore the possibility that the SNP6 may differentially bind to nuclear proteins, probes containing the SNP6 A allele or the G allele were incubated with the nuclear extracts from human T lymphocytes stimulated with interleukin (IL)-4 in an electrophoretic mobility-shift assay (EMSA). A 1.85-fold or 1.75-fold increase in the band density was observed for the SNP6 G allele in comparison with the common A allele from two independent experiments, although no pattern changes were observed (Figure 5). A similar but weaker result was obtained in the IL-2 stimulated T cells (unpublished data). This suggests that the SNP6 G allele has higher binding affinity with unknown nuclear proteins.

Discussion

The ability of Apobec3G to restrict HIV-1 replication through hypermutation is suppressed by HIV-1 Vif binding to CUL5 gene product Cullin 5, leading to the polyubiquitination and degradation of Apobec3G and Apobec3F through the Cullin 5-Elongin B-Elongin C E3 ubiquitin ligase pathway [7,14,21]. To understand the role of genetic variation in the gene encoding Cullin 5 on HIV-1/AIDS, we examined 12 SNPs and their haplotypes on risk and progression of HIV-1 disease. The haplotypes formed two clusters defined by alleles at the proxy SNPs 3, 5, and 8. Relative to cluster I, cluster II haplotypes as a group were associated with faster CD4 T cell decline as indicated by the Cox model analysis of survival to the endpoint of CD4 < 200 and reaffirmed by the mixed effects model assessing CD4 T cell trajectory over time. Individuals who were carriers of any two cluster II haplotypes progressed to CD4 < 200 about 5.7 years faster than those who carried any two cluster I haplotypes. The effect of cluster II was largely attributable to Hap10, carrying both ctSNP5 G and htSNP6 G alleles. On the other hand, cluster I contains the two protective haplotypes, Hap1 and Hap6, which delayed CD4 T cell loss. We also observed an additive interaction between CUL5 cluster II haplotypes and APOBEC3G-H186R on CD4 T cell slope, suggesting that Apobec3G and Cullin 5 likely confer independent effects on CD4 T cell loss [18].

The genetic effects derived from multiple SNPs and haplotypes, and from the haplotype clusters, provide evidence that genetic variation in CUL5 likely modifies the rate of disease progression of HIV-1 with the effects being stronger and more consistent among AA than EA. The differences observed between the two racial groups suggest that there are additional functional alleles in LD with the SNP markers assessed in this study.

To assess the possibility that the observed associations were due to the multiple tests, we estimated the FDR for all the tests performed. Although this test is quite conservative as there is considerable correlation among SNPs, haplotypes, and disease endpoints, the FDR for significant (p < 0.05) SNP and haplotype associations in both AA and EA was 5% or less. However, the gold standard for validation of genetic associations remains confirmation in other adequately powered studies.

Human Cullin 5 is a highly conserved 780-amino acid protein, differing by only seven amino acids from the rabbit VACM-1 protein [22,23]. The conservation of Cullin 5 amino acid sequence suggests that the Cullin 5 protein is under strong functional constraint and purifying selection. The fact that genetic effects observed for SNP5 and SNP6 were most robust under the additive genetic model suggests a dose-effect of the factors. It is thus most likely that the causal sequence or alleles lie in regulatory elements affecting CUL5 mRNA or protein levels. However, no promoter or non-synonymous SNPs were reported among over 230 SNPs in the CUL5 gene deposited in the dbSNP database as of July 6, 2006.
| SNP Genotype | Genetic Model | From Seroconversion to July 1997<sup>a</sup> | From Seroconversion to July 2004<sup>b</sup> |
|--------------|---------------|--------------------------------------------|--------------------------------------------|
|              | Number of Individuals | Number of Measurements | CD4<sup>+</sup> T Cell Slope (95% CI) | p   | q     | Number of Measurements | CD4<sup>+</sup> T Cell Slope (95% CI) | p   | q     |
| CUL5 SNPs    | AA            | 94             | 754             | -0.99 (-1.99, -0.001) | 0.05 | 0.076 | 1276             | -1.23 (-2.20, -0.26) | 0.01 | 0.026 |
|              | AG            | 124            | 923             | Additive: GG > AG > AA | 0.05 | 0.076 | 1624            | -0.03 (-1.51, 1.45) | 0.86 | 0.132 |
|              | GG            | 36             | 285             | 0.02 (0.00, 0.04) | 0.68 | 1.000 | 471             | 0.12 (0.03, 0.21) | 0.05 | 0.014 |
|              | Additive: GG > AG > AA | 0.05 | 0.076 | 1624            | -0.03 (-1.51, 1.45) | 0.86 | 0.132 |
| CUL5 SNPs    | AA            | 231            | 1779            | -2.56 (-4.49, -0.53) | 0.01 | 0.026 | 3063            | -2.63 (-4.64, -0.61) | 0.01 | 0.026 |
|              | AG            | 21             | 165             | 0.12 (-0.16, 0.40) | 0.26 | 0.394 | 287             | -0.03 (-1.51, 1.45) | 0.86 | 0.132 |
|              | GG            | 2              | 18              | 0.02 (0.00, 0.04) | 0.68 | 1.000 | 21              | 0.12 (0.03, 0.21) | 0.05 | 0.014 |
|              | Additive: GG > AG > AA | 0.05 | 0.076 | 1624            | -0.03 (-1.51, 1.45) | 0.86 | 0.132 |
| APOBEC3G    | HH            | 89             | 742             | 1.25 (2.65, 0.15) | 0.08 | 0.001 | 1256            | 1.41 (2.65, 0.15) | 0.08 | 0.001 |
|              | HR            | 124            | 928             | 0.11 (0.01, 0.22) | 0.07 | 0.851 | 1594            | 0.12 (0.01, 0.22) | 0.07 | 0.851 |
|              | RR            | 39             | 296             | 0.12 (0.01, 0.22) | 0.07 | 0.851 | 511             | 0.12 (0.01, 0.22) | 0.07 | 0.851 |
|              | Dominant: HR or RR | —             | —              | —             | —     | —     | —              | —             | —     | —     |
|              | Additive: HR > RR > HH | —             | —              | —             | —     | —     | —              | —             | —     | —     |
| APOBEC3G/CUL5 SNPs | HH/AA         | 79             | 652             | 3.20 (5.49, 0.92) | 0.01 | 0.026 | 1101            | 3.31 (5.49, 0.92) | 0.01 | 0.026 |
|              | HR or RR/AG or GG | 99             | 722             | 0.12 (0.01, 0.22) | 0.07 | 0.851 | 424             | 0.12 (0.01, 0.22) | 0.07 | 0.851 |
|              | APOBEC3G/CUL5 SNPs | 99             | 722             | 0.12 (0.01, 0.22) | 0.07 | 0.851 | 424             | 0.12 (0.01, 0.22) | 0.07 | 0.851 |
|              | HH/AA         | 79             | 652             | 3.20 (5.49, 0.92) | 0.01 | 0.026 | 1101            | 3.31 (5.49, 0.92) | 0.01 | 0.026 |
|              | HR or RR/AG or GG | 14             | 99              | 3.64 (7.18, 1.13) | 0.02 | 0.043 | 160             | 4.16 (7.18, 1.13) | 0.007 | 0.026 |
|              | —             | —              | —              | —             | —     | —     | —              | —             | —     | —     |
|              | APOBEC3G/CUL5 SNPs | 99             | 722             | 0.12 (0.01, 0.22) | 0.07 | 0.851 | 424             | 0.12 (0.01, 0.22) | 0.07 | 0.851 |
|              | HH/AA         | 79             | 652             | 3.20 (5.49, 0.92) | 0.01 | 0.026 | 1101            | 3.31 (5.49, 0.92) | 0.01 | 0.026 |
|              | HR or RR/AG or GG | 14             | 99              | 3.64 (7.18, 1.13) | 0.02 | 0.043 | 160             | 4.16 (7.18, 1.13) | 0.007 | 0.026 |
|              | —             | —              | —              | —             | —     | —     | —              | —             | —     | —     |
| CUL5 SNPs    | AA            | 94             | 667             | 0.12 (0.01, 0.22) | 0.07 | 0.851 | 1193            | 0.12 (0.01, 0.22) | 0.07 | 0.851 |
|              | AG            | 124            | 805             | 0.12 (0.01, 0.22) | 0.07 | 0.851 | 1513            | 0.12 (0.01, 0.22) | 0.07 | 0.851 |
|              | GG            | 36             | 242             | 0.12 (0.01, 0.22) | 0.07 | 0.851 | 242             | 0.12 (0.01, 0.22) | 0.07 | 0.851 |
|              | Additive: GG > AG > AA | 0.05 | 0.076 | 1624            | -0.03 (-1.51, 1.45) | 0.86 | 0.132 |
| CUL5 SNPs    | AA            | 231            | 1553            | 0.18 (0.01, 0.36) | 0.04 | 0.068 | 2854            | 0.12 (-0.03, 0.27) | 0.12 | 0.12  |
|              | AG            | 21             | 146             | 0.12 (-0.03, 0.27) | 0.12 | 0.12  | 270             | 0.12 (-0.03, 0.27) | 0.12 | 0.12  |
|              | GG            | 2              | 15              | 0.12 (-0.03, 0.27) | 0.12 | 0.12  | 18              | 0.12 (-0.03, 0.27) | 0.12 | 0.12  |
|              | Additive: GG > AG > AA | 0.05 | 0.076 | 1624            | -0.03 (-1.51, 1.45) | 0.86 | 0.132 |

Tests were done by the mixed-effects model with adjustments for age and sex; CD4<sup>+</sup> T cell counts were square-root transformed, and HIV-1 RNA levels were log<sub>10</sub> transformed.

<sup>a</sup>Observation period was from seroconversion to July 1997 before the onset of HAART.

<sup>b</sup>From seroconversion to July 2004.

CI, confidence interval; p, p-values; q, q-values (measures for the FDR)

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Table 3. Effects of CUL5 and APOBEC3G SNPs on the Longitudinal CD4<sup>+</sup> T cell and HIV-1 RNA Slope Gradients in the ALIVE Cohort
CUL5 Polymorphisms and AIDS

Study participants. Study participants were enrolled in five United States-based natural history HIV/AIDS cohorts. ALIVE is a community-based cohort of intravenous injection drug users in Baltimore enrolled in 1988–1989 [24], consisting of 92% AA. Multicenter AIDS Cohort Study (MACS) is a longitudinal prospective cohort of men who have sex with men from four U.S. cities: Chicago, Baltimore, Pittsburgh, and Los Angeles enrolled in 1984–1985 [25], consisting of 83% EA and 10% AA. The San Francisco City Clinic Study (SFCC) is a cohort of men who have sex with men originally enrolled in a hepatitis B study in 1978–1980 [26], consisting of 96% EA. Hemophilia Growth and Development Study (HGDS) is a multicenter prospective study that enrolled children with hemophilia who were exposed to HIV-1 through blood products between 1982 and 1983 [27], consisting of 72% EA and 11% AA. The Multicenter Hemophilia Cohort Study (MHCS) is a prospective study that enrolled persons with hemophilia [28], consisting of 90% EA and 6% AA. The individuals genotyped in this report consisted of HIV-1 SC, seroprevalents, SN, and high-risk exposed uninfected (HREU) for a total of 3,476 participants (2,169 EA and 1,307 AA). The numbers of EA and AA individuals studied in each disease category were as follows: SC = 659, 290; SN = 309, 336; HREU = 141, 82, respectively. Of 290 AA SC, 237, 42, five, and five were from ALIVE, MACS, MHCS, and HGDS, respectively.

The date of seroconversion after study enrollment was estimated as the midpoint between the last seronegative and first seropositive HIV-1 antibody test; only individuals with less than two years’ elapsed time between the two tests were included in the seroconverter group for analysis. The censoring date was the earliest of the date of the last recorded visit, or December 31, 1995 for the MACS, MHCS, HGDS, and SFCC, or July 31, 1997 for the ALIVE cohort, to avoid potential confounding by HAART. The censoring date was extended in the ALIVE cohort because of delayed administration of HAART to this group [24,29]. The MACS, MHCS, SFCC, and ALIVE consists of both SC (infected after study enrollment) and seroprevalents (infected before study enrollment) individuals; because of the potential for frailty bias (missing the most rapid progressors to AIDS and death) among seroprevalents, only SC enrolled in the ALIVE, MACS, MHCS, and SFCC were used in the analysis. In addition, DNA samples from 110 normal blood donor HC were included to provide an estimate of allele frequencies in a major Asian population and to inform for future CUL5 genetic studies in this population. This group was not used in the association analyses.

The study was approved by the Institutional Review Boards of participating institutes, and informed consent was obtained from the participants.

Identification of SNPs. A panel consisting of 94 DNA samples each from EA and AA was partially resequenced to discover novel CUL5 polymorphisms. PCR primers were designed based on GenBank DNA sequence AP003307 and mRNA sequence NM_003478 to cover the putative promoter region, 5’ UTR, exons 15–19 encoding the C terminus that was shown to confer major function [7], and 3’ UTR of the CUL5 gene. SNPs were obtained from dbSNP, HapMap (http://www.hapmap.org), and TaqMan SNP Genotyping Assay databases (http://www.appliedbiosystems.com/). A total of 12 SNPs were selected for genotyping by considering SNP location, spacing, and at least 5% allele frequency (Figure 1A and 1B). Haplotype-tagging (ht)SNPs were given preference in the SNP selection.

Genotyping of SNPs. Genotyping was performed using TaqMan assays according to the manufacturer’s manual (Applied Biosystems, http://www.appliedbiosystems.com/). TaqMan primer and probes were designed by using the Primer Express software or by the Assay-by-Demand service of Applied Biosystems (Table S1). A total of eight water controls were included on each plate to monitor the potential PCR contamination, and 10% of SC and HREU samples were genotyped twice. The genotypes obtained were free of water contamination or of inconsistencies between duplicates. SNP5 was genotyped twice using two different sets of primers and probes, and the results were identical.

Materials and Methods

Figure 5. EMSA Analysis of CUL5 SNP6 A/G

Nuclear extracts from human T lymphocytes induced by IL-4 were bound to the oligonucleotide containing the SNP6 A allele (lane A) or G allele (lane B), without cold competitors. Lane C contained nuclear extracts, SNP6 A allele probe, and a 100-fold excess of its cold probe as competitor. Lane D contained nuclear extracts, SNP6 G allele probe, and a 100-fold excess of its cold probe as competitor. An arrow indicates the band showing differential binding of nuclear factor(s) to the oligonucleotide.
Statistical analysis. Analyses were conducted using the statistical packages SAS version 9.0 (SAS Institute, http://www.sas.com). Conformity to the genotype frequencies expected under Hardy-Weinberg equilibrium was examined for each SNP. The genetic effects of SNPs on HIV-1 infection susceptibility were assessed by comparing allelic frequencies between HIV-1 HREU and HIV-1 SC participants using the Fisher exact test. Pairwise linkage disequilibrium and haplotype structure were determined using the program Haploview (www.broad.mit.edu/mpg/haploview) [44]. A triangular matrix of $D^9$ statistic was used to demonstrate LD patterns within AA and EA. Haplotype blocks were defined with a default algorithm based on the confidence intervals of $D^9$. Haplotypes were inferred by the PhASE program, and RH was determined by unadjusted and adjusted Cox model regression analyses. For each SNP, we compared the minor allele genotypes to the most common genotype as a reference group. To determine if there was an additive effect of SNPs or alleles, the additive genetic model was tested by comparing survival in persons carrying two to one minor alleles to the homozygous reference group, coded as 2, 1, and 0, respectively, in the regression analysis: RH reflects effects of each copy of the allele or the haplotype. All $p$-values were 2-tailed. Genetic factors previously shown to affect progression to AIDS and AA groups were included as confounding covariates in the adjusted Cox model analysis: CCR5 $\Delta32$ [32]; CCR2-64I [33]: HLA-B*27 [35]; HLA-B*5703 group (including HLA-B*5702, B*5703, B*5704, and B*5701) [36]; and HLA class I homozygosity [57] for EA; HLA-B*57 and HLA class I homozygosity for AA. CCR2-64I, HLA-B*27, and HLA-B*5703 groups were not considered as covariates in AA due to absent or weak effects in our AA participants, and CRR5 $\Delta32$ was not considered due to its rarity in AA. Participants were stratified by sex and by age at seroconversion: 0–20, >20–40, and >40 years [38].

Supporting Information

Table S1. Genotyping Methods for CUL5 SNPs

Table S2. Cox Model Analysis of CUL5 Haplotypes

Table S3. Selection of the Top Models for CUL5 Haplotypes Using AIC

Table S4. Cox Model Analysis of CUL5 SNPs in EA

Accession Numbers

The Entrez Gene database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene) accession numbers for the genes discussed in this paper are Cullin 5 (8065), Elongin B (6923), Elongin C (6921), Rbaf (9578), Apoer3G (60489), Apoer3F (200316), and HIV-1 Vif (155459).

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4. EMMA. Cell culture and EMMA were performed as described [46,47]. Freshly explanted human T lymphocytes were obtained from normal donors, purified by isoelectrophoresis, and activated for 72 h with 1 mg/ml phytohemagglutinin (PHA) in RPMI 1,640 medium containing 10% fetal calf serum (FCS) (Sigma, http://www.sigma-aldrich.com) 2 mM L-glutamine, and penicillin-streptomycin (50 IU/ml and 50 mg/ml, respectively). T lymphocytes were made quiescent by washing and incubating for 24 h in RPMI 1,640 medium containing 1% FCS before exposure to cytokines. Cells were then stimulated with 100 nM IL-4 (PeproTech, http://www.peprotech.com) or 100 nM human IL-2 (Hoffmann-La Roche, http://www.rocheusa.com) at 37 °C for 10 min. Cell pellets were frozen at −70 °C. The probe sequences were 5′-CAGTGGACATACCTGTGTTAGG-3′ for SNP6 A allele and 5′-CAGTGGACATCCCTGTGTTAGGA-3′ for SNP6 G allele. In cold oligonucleotide competition assay, 100-fold excess of unlabeled probe was added as a competitor. The band density was measured by the software ImageJ (http://rsb.info.nih.gov/ij/index.html).

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