Modulating influence on HIV/AIDS by interacting *RANTES* gene variants

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RANTES (regulated on activation normal T cell expressed and secreted), a ligand for the CC chemokine receptor 5, potently inhibits HIV-1 replication *in vitro*. We tested the influence of four *RANTES* single nucleotide polymorphism (SNP) variants and their haplotypes on HIV-1 infection and AIDS progression in five AIDS cohorts. Three SNPs in the *RANTES* gene region on chromosome 17 (*403A* in the promoter, *In1.1C* in the first intron, and *3′222C* in the 3′ untranslated region) are associated with increased frequency of HIV-1 infection. The common *In1.1C* SNP allele is nested within an intronic regulatory sequence element that exhibits differential allele binding to nuclear proteins and a down-regulation of gene transcription. The *In1.1C* allele or haplotypes that include *In1.1C* display a strong dominant association with rapid progression to AIDS among HIV-1-infected individuals in African-American, European-American, and combined cohorts. The principal *RANTES* SNP genetic influence on AIDS progression derives from the down-regulating *RANTES In1.1C* allele, although linkage disequilibrium with adjoining *RANTES* SNPs including a weaker up-regulating *RANTES* promoter allele (*−28G*), can modify the observed epidemiological patterns. The *In1.1C*-bearing genotypes account for 37% of the attributable risk for rapid progression among African Americans and may also be an important influence on AIDS progression in Africa. The diminished transcription of *RANTES* afforded by the *In1.1C* regulatory allele is consistent with increased HIV-1 spread *in vivo*, leading to accelerated progression to AIDS.

The entry of HIV-1 into $CD4^+$ cells is mediated by interactions between the viral envelope glycoproteins, the CD4 receptor, and HIV-1 coreceptors. The primary HIV-1 coreceptors are the chemokine receptors CCR5 (CC chemokine receptor 5), used by R5 HIV-1 strains, and CXCR4, used by X4–HIV-1 strains that emerge during the later stages of infection. The CCR5 ligands, RANTES (regulated on activation normal T cell expressed and secreted), MIP-1α, and MIP-1β, and the CXCR4 ligand SDF-1α all are potent inhibitors of HIV-1 cell entry and replication (1). Variants in the HIV-1 coreceptors and their natural ligand genes have been shown to modify HIV-1 transmission and disease progression (2–9).

RANTES inhibits CCR5-mediated entry of R5 strains by competitive binding and down-modulation of CCR5 (10, 11). HIV-1-exposed, but uninfected, individuals produce high levels of RANTES from peripheral blood mononuclear cells or cultured $CD4^+$ T cells (12–14), and in HIV-1-infected individuals, those with higher levels of RANTES postpone the onset of AIDS-defining pathologies (refs. 15–19; reviewed in ref. 20).

Two single nucleotide polymorphism (SNP) sites, *−28C/G* and *−403G/A*, in the promoter region of *RANTES* have been identified (8). The *−28G* variant, but not *−403A*, was reported to up-regulate *RANTES* transcription in one study (8) whereas *−403A* was reported to up-regulate *RANTES* transcription in a second study without consideration of *−28C/G* (21). The [*−403A−28G*] haplotype was shown to be associated with a slower rate of $CD4^+$ T-cell depletion in HIV-1-infected Japanese (8). In European Americans (EA) the compound genotype [*−403G/A−28C/C*] was reported to be susceptible to HIV-1 infection but resistant to AIDS progression when compared with genotype *−403G/G−28C/C* in one study (9), but to be susceptible to both HIV-1 infection and AIDS progression in another (22). No effect on HIV-1 infection and AIDS progression by these variants has been reported in African Americans (AA) (22).

Considering the potential interaction of these two *RANTES* gene polymorphisms and the complex nature of *RANTES* gene expression (8, 9, 21–24), we reasoned that it would be valuable to screen the entire *RANTES* gene region for nucleotide polymorphisms and to perform an infection and survival association analysis on the same five cohorts that have been used to discover eight AIDS restriction genes (25, 26). Here we describe a group of seven SNPs within the *RANTES* gene including one, *In1.1T/C*, within a newly identified intronic *RANTES* regulatory element that modulates *RANTES* transcription, possibly influences HIV-1 infection, and affects the rate of progression to AIDS in HIV-1-infected individuals.

Materials and Methods

Study Population. The study group includes 964 seroconverters, 2,103 seroprevalents, and 1,101 seronegatives for a total of 4,168 (EA, 2,594; AA, 1,574) from five natural history longitudinal AIDS cohorts: AIDS Link to the Intravenous Drug Experience (27), Hemophilia Growth and Development Study (28), Multicenter AIDS Cohort Study (29), Multicenter Hemophiliac Cohort Study (30), and the San Francisco City Clinic Study (31). An additional 129 Han Chinese normal blood donors were genotyped for allele frequencies. Informed consent was obtained from all study participants. Seroconversion date was estimated as the midpoint between the last seronegative and the first seropositive HIV-1 antibody test date (mean interval 0.79 years, range 0.07 to 3.0 years). High-risk exposed uninfected subjects ($n = 271$) were those with high-risk exposure through sharing of injection equipment (32), anal receptive sex with multiple partners (33), or transfusions with factor VIII clotting factor before 1984 when heat treatment was initiated (34).

Identification of DNA Polymorphisms. To identify nucleotide polymorphisms in *RANTES* we used a DNA panel consisting of 72 EA and 72 AA. A nonisotopic RNA cleavage assay (NIRCA) was performed to screen polymorphisms by using the Mismatch Detect II kit (Ambion, Austin, TX) according to the manufacturer's instructions (35). Overlapping PCR primers were designed to cover the entire *RANTES* gene except a portion of intron 2 (positions 166995 to 170610 and 176293 to 177383 of the GenBank sequence

Abbreviations: *RANTES*, regulated on activation normal T cell expressed and secreted; CCR5, CC chemokine receptor 5; SNP, single nucleotide polymorphism; EA, European Americans; AA, African Americans; OR, odds ratio; RH, relative hazard.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF336300 and AF336301).

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AF088219). Primer sequences and PCR conditions are available from the authors. PCR products that revealed aberrant bands by NIRCA analysis were purified and sequenced.

Electrophoretic Mobility-Shift Assay (EMSA). Nuclear protein extraction and EMSA were performed as described (36). $CD4^+$-enriched lymphocytes were obtained from human T cells by high affinity negative selection with a human T cell CD4 Subset Column Kit (R&D Systems). After activation with 5 μg/ml phytohemagglutinin for 7 days in the presence of 20 units/ml IL-2, $CD4^+$-enriched lymphocytes were harvested for preparing nuclear extracts. The probe sequences were 5′-gatcagtttttctgtctt**t**aaggtctacaccctcaa-3′ for *In1.1T*, and 5′-gatcagtttttctgtctt**c**aaggtctacaccctcaa-3′ for *In1.1C*. The probes were filling-labeled with ^{32}P-dATP.

Cloning of the Intron 1 Fragment. PCR products of intron1 fragments spanning nucleotides 168693 to 169104 were obtained by using primers RT-In-F-*Bam*HI 5′-tcaaggatccgtaagtcctggtcttgaccacc-3′ and RT-In-R-*Sal*I 5′-acgcgtcgacgaatatggctgtctcagggtct-3′ and Turbo *Pfu* polymerase (Stratagene) and were subsequently placed into the *Bam*HI and *Sal*I sites in pGL3-promoter vector (Promega).

Haplotype Cloning of the Intron 1 and Promoter Fragments. *RANTES* promoter fragments spanning −634 to +45 bp [numbering according to Liu *et al.* (8)] containing *−403G/A −28C/G* were amplified from *RANTES* promoter haplotype vectors (8) (provided by Tatsuo Shioda, University of Tokyo) with Turbo *Pfu* polymerase, and subsequently placed into the *Mlu*I and *Xho*I sites upstream of the *Luc+* gene in the pGL3-basic vector (Promega), whereas the 412-bp intron 1 fragments with *In1.1T* or *In1.1C* were placed into the downstream *Bam*HI and *Sal*I sites. All constructs were verified by sequencing.

Transfection and Luciferase Assays. Transient transfections of Jurkat cells were performed as described (36). Cationic liposomes (Roche Molecular Biochemicals) were used to transfect the reporter plasmids into Jurkat cells. After stimulation by phytohemagglutinin/phorbol 12-myristate 13-acetate for 24 h at 37°C, cells were lysed and measured for luciferase activity in the Luciferase Assay System (Promega). Luciferase activity was normalized against protein concentration.

Genotyping of *RANTES* Polymorphisms. Two methods, PCR-restriction fragment length polymorphism (PCR-RFLP) and 5′ nuclease PCR assays (TaqMan), were used for genotyping. SNPs *−28C/G*, *In1.1T/C*, and *3′222T/C* were genotyped by PCR-RFLP with primers and endonuclease restriction enzymes as follows: *−28C/G*, forward, 5′-actcgaatttccggaggcta and reverse, 5′-tctgcagctcaggctggccctttat using *Mnl*I for digestion; *In1.1T/C*, forward, 5′-cctggtcttgaccaccaca and reverse, 5′-gctgacaggcatgagtcaga using *Mbo*II for digestion; and *3′222T/C*, 5′-ctgtcccggtactgacaagg and 5′-cccgagtagctgggactaca using *Hph*I for digestion. SNPs −105 and −109 were genotyped by forced PCR-RFLP with primers RT(−105)F 5′-ttggtgcttggtcaaagagg and RT(−105)R 5′-ccggtatcataagtgaaattcca using *Bsl*I for digestion and RT(−109)F 5′-ggtgcttggtcaaagaggaa and RT(−109)R 5′-catggtacctgtggaagagg using *Ear*I for digestion, respectively. The artificially introduced nucleotides are underlined. All restriction enzymes were purchased from New England Biolabs.

TaqMan assays were performed by using PCR primers and TaqMan probes (Perkin–Elmer) in the ABI Prism 7700 Sequence Detector (Applied Biosystems) according to the manufacturer's manual. SNP *−403G/A* was genotyped with primers RT(*−403*)TBF 5′-tccagaggaccctcctcaataa and RT(*−403*)TBR 5′-ctgagtcactgagtcttcaaagttcc and MGB TaqMan probes RT(*−403*)MGB-G 5′-FAM-aaaggagg**g**taagatctgtaat and RT(*−403*)MGB-A 5′-VIC-aaaggaga**t**aagatctgtaatg. SNP *In1.2T/C* was genotyped by using primers RT(*In1.2*)-TMF 5′-gcctcagtttctgtcaaggaaga and RT(*In1.2*)-TMR 5′-agggagacccttttattcattgc and the TaqMan probes RT(*In1.2*)-TM-G 5′-FAM-tgtccagcacaat**g**tcaagtgtgca and RT(*In1.2*)-TM-A 5′-VIC-aagtgtccagcacaat**a**tcaagtgtgcagta. SNP sites are in bold. Detailed protocols are available from the authors.

Statistical Analysis. Haplotypes were inferred by the expectation maximization algorithm (37). Kaplan–Meier nonparametric survival statistics and the Cox proportional hazards model in the SAS package (SAS Institute, Cary, NC) were used for survival analysis. Three endpoints reflecting advancing HIV-1 disease progression were evaluated: time to less than 200 $CD4^+$ cells per mm^3 (CD4 <200); AIDS-1987, as defined by the Centers for Disease Control and Prevention (38); and AIDS-related death. The censoring date was the earliest of the date of the last recorded visit, or July 31, 1997 (for the AIDS Link to the Intravenous Drug Experience cohort), or December 31, 1995 (for all other cohorts). Cox model analyses were performed both unadjusted (using the haplotype factor by itself) and adjusted (considering the *RANTES* haplotype factor as a covariate along with additional AIDS-modifying genetic factors: for EA, *CCR5-Δ32*, *CCR2–64I*, *SDF1–3′A*, *HLA-B*27*, *HLA-B*57*, *HLA-B*35Px*, and *HLA* class I zygosity; for AA, *HLA-B*57*, *HLA-B*35Px*, and *HLA* class I zygosity (2–5, 7, 25, 26, 39). Participants were stratified by ethnic group and sex (7.6% female) and age group at seroconversion (0–20, >20–40, and over 40 years).

Defined categorical analyses (DCA) compared seroconverters progressing more rapidly than the median progression time to the specified outcome to seroconverters and seroprevalents surviving outcome free for at least that long; for AA a shorter time of 7.5 years was used for AIDS-1987 and death, to allow for the relatively short follow-up time for this group. The two-tailed Fisher's exact test was performed for the DCA and infection tests, except that the codominant model was tested by the Mantel–Haenszel χ^2, and Cochran–Mantel–Haenszel stratified analysis was used for combined EA and AA. The attributable fraction (AF) was computed by the formula $AF = f(R-1)/[1 + f(R-1)]$, where f is the frequency of the risk factor in the population and R is the measure of relative risk (40); relative risk was obtained from a DCA using only seroconverters. The unpaired t test was used to assess the difference in transcription activities in the gene reporter assay.

Results

Polymorphisms and Linkage Disequilibrium Within the *RANTES* Gene. The human *RANTES* gene spans 8.8 kb on chromosome 17q11.2-q12 and has the characteristic three exon–two intron organization of the CC chemokine family. Nonisotopic RNA cleavage assay and DNA sequencing were used to detect SNPs in the entire *RANTES* gene except a part of intron 2. Seven SNPs were identified (Fig. 1*a*): four in the promoter, including the previously described *−403G/A* and *−28C/G* (8), two in the first intron, and one in the 3′ untranslated region. The genotype of each SNP was determined for 4,168 individuals enrolled in five HIV-AIDS prospective cohorts plus 129 Han Chinese Asians. The *−403A* promoter allele was common in all populations tested with allele frequencies (f) of 0.18 in EA, 0.43 in AA, and 0.36 in Asians. The *−28G* promoter allele was infrequent in all groups except Asians ($f = 0.13$). Two rare promoter SNPs, *−105C/T* and *−109T/C*, were located within the five IFN-simulated response elements between positions −124 and −97. Two SNPs were found in intron 1, *In1.1T/C* and *In1.2G/A*. *In1.1C* was frequent in all groups ($f = 0.14$ in EA, 0.20 in AA, and 0.34 in Asians), whereas *In1.2A* was infrequent in AA ($f = 0.06$) and absent in other ethnic groups. SNP *3′222T/C*, located in an *Alu*-related repeat region of 3′ untranslated region, had a frequency of 0.11 in EA, 0.07 in AA, and 0.24 in Asians. Three rare SNPs (*105C/T*, *109C/T*, and *In1.2G/A*) were not considered further.

Strong linkage disequilibrium was observed between all of the *RANTES* SNPs, and in particular between the alleles at the four

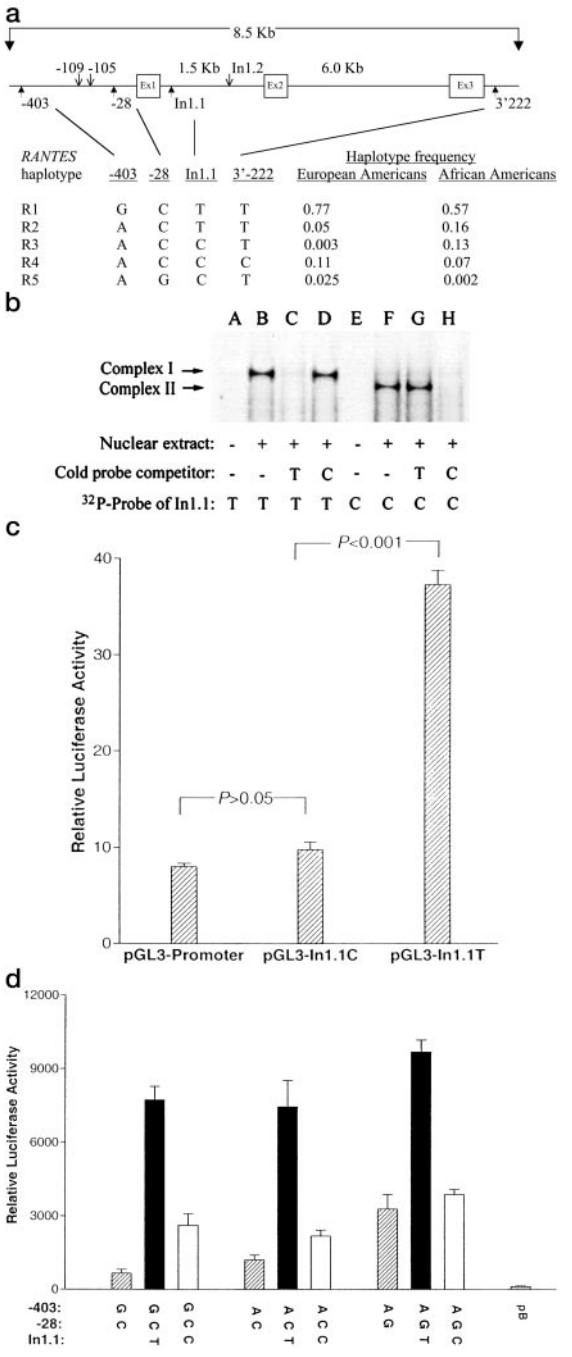

Fig. 1. (*a*) The genomic structure of the *RANTES* gene on chromosome 17q11.2 and the haplotype structure and frequency of the *RANTES* variants. Exons are shown as open boxes, and intron sizes are indicated. Locations of nucleotide variants are indicated by arrows. Variants in the promoter region are numbered according to the transcription start site, and the SNP in the 3′ untranslated region is numbered from the first nucleotide of the 3′ untranslated region. Variants *In1.1* and *In1.2* are located at nucleotide positions 168923 and 170226, respectively, in GenBank sequence AF088219. (*b*) Competitive electrophoretic mobility-shift assay of DNA–nuclear protein binding at the *In1.1T/C* site. Nuclear extracts from $CD4^+$-enriched lymphocytes bound to the intron 1 fragment containing *In1.1T* (lanes A–D) or *In1.1C* (lanes E–H). Lanes A and E, no extract; lanes B and F, *In1.1T* and *In1.1C* probe, respectively, without competitors; lanes C and G, *In1.1T* and *In1.1C* probe, respectively, with a 100-fold excess of cold *In1.1T* probe as competitor; lanes D and H, *In1.1T* and *In1.1C* probe, respectively, with a 100-fold excess of cold *In1.1C* probe as a competitor. The arrowheads indicate the specific DNA–protein complexes associated with *In1.1T/C*. (*c*) Luciferase activities of pBL3-promoter constructs containing *RANTES* intron 1 fragments with either *In1.1T* (pGL3-*In1.1T*) or *In1.1C* (pGL3-*In1.1C*). The pBL3-promoter vector served as a control. Bars indicate mean values with standard deviations; *P* values were determined by Student's *t* test. Results are the mean of two experiments performed in triplicate. (*d*) Luciferase activities of the *RANTES* haplotype constructs containing the *RANTES* promoter (*−403G/A*, *−28C/G*) and the intron 1 element (*In1.1T/C*) allele combinations. The pGL3-basic vector constructs contain the promoter fragments only (hatched bar), promoter and intron 1 fragments carrying *In1.1T* (black bars), and promoter and intron 1 fragments carrying *In1.1C* (dotted bars). Haplotypes are denoted by the nucleotide at positions −403, −28, and In1.1, respectively. These constructs were also tested in the U937 monocyte cell and CCRF-SB B cell lines. The gene transcription activities were similar in the U937 cells but were much weaker in the CCRF-SB B cells, compared with those in Jurkat T cells (data not shown). The pBL3-basic vector (pB) served as a control. Note: haplotype *AGT* has not been observed in the population studied.

most polymorphic sites, *−403*, *−28*, *In1.1*, and *3′222* ($0.96 < D' < 1$, $P < 10^{-6}$). Five of the 16 possible *RANTES* haplotypes (R1–R5, Fig. 1*a*) for these four SNP sites accounted for more than 98% of EA and AA chromosomes (Fig. 1*a*). Within the common haplotypes, *In1.1C* occurs always on a haplotype containing *−403A*, whereas *−28G* and *3′222C* always occur on the haplotype containing *−403A* and *In1.1C* variant alleles, but SNPs *−28G* and *3′222C* never occur together. In both races, the majority of individuals carry the common haplotype R1 (57.0% and 77.4% in AA and EA, respectively).

***RANTES In1.1C* Occurs in an Up-Regulating Intron 1 Element.** Two promoter SNPs, *−28G* and *−403A*, have been reported to up-regulate *RANTES* transcription (8, 21). We investigated the role of *In1.1C* by screening nuclear extracts for proteins that would bind alternative alleles and by quantifying their influence on *RANTES* gene transcription in luciferase gene expression constructs. Using an electrophoretic mobility-shift assay synthetic (36-bp) oligonucleotide probes containing *In1.1C* were incubated with nuclear extracts from human $CD4^+$-enriched lymphocytes. Two distinctive complexes, I and II, were observed with probes containing *In1.1T* and *In1.1C*, respectively (Fig. 1*b*). Specificity of the binding was confirmed by cross-competition with unlabeled *In1.1T* or *In1.1C* probes: competitive binding of *In1.1T*, but not *In1.1C*, eliminated complex I, and correspondingly, competition with *In1.1C*, but not *In1.1T*, eliminated complex II. These results demonstrate that the *In1.1T/C* alleles bind to different nuclear proteins or different forms of the same protein.

To assess the role of the *In1.I.T/C* alleles on gene transcription, a *RANTES* intron 1 fragment containing either *In1.1T* or *In1.1C* was inserted downstream of the luciferase (luc^+) gene in a simian virus 40 promoter-containing construct and tested in Jurkat T cells. The construct containing *In1.1C* showed a 3-fold reduction in gene expression relative to the construct containing *In1.1T* ($P < 0.001$, Fig. 1*c*) demonstrating a down-regulating role for *In1.1C* on gene transcription.

We next tested the transcriptional effects of combinations of the *RANTES* promoter (*−403G*/C, *−28C*/G) and the putative intronic (*In1.1T*/C) regulatory element alleles on gene transcription by placing the *RANTES* promoter and intron 1 fragments upstream and downstream, respectively, of the luc^+ gene. The constructs containing the *In1.1T* intron 1 fragment up-regulated *RANTES* transcriptional activity for the three promoter-intron constructs (denoting the haplotype as the nucleotide at positions −403, −28, and In1.1) *GCT* (12-fold, $P < 0.001$), *ACT* (6-fold, $P < 0.001$), and *AGT* (3-fold, $P < 0.001$) relative to the promoter-alone constructs (positions −403 and −28) *GC*, *AC*, and *AG*, respectively (Fig. 1*d*). However, relative to the *In1.1T* allele, *RANTES* transcriptional activity was reduced by 2.5-to 3.4-fold in similar constructs containing the variant *In1.1C* allele: *GCC* (3-fold, $P = 0.002$), *ACC* (3.4-fold, $P = 0.009$), or *AGC* (2.5-fold, $P < 0.001$). These results demonstrate both that the intron 1 fragment is a strong regulatory

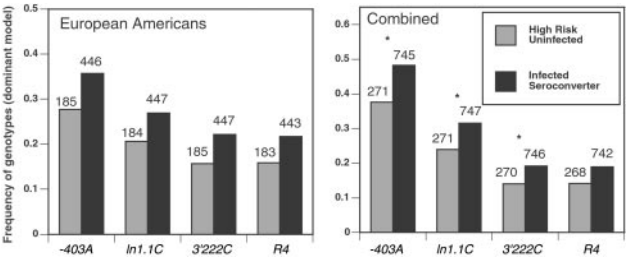

Fig. 2. Comparison of frequency of genotypes carrying *RANTES* SNPs or the R4 haplotype between high-risk HIV-1-exposed but uninfected and HIV-1 seroconverters for EA (*Left*) and combined EA and AA (*Right*). Numbers above bars are the numbers of subjects in each group; * indicates a significant frequency difference (dominant model) by a two-sided Fisher's exact test.

element and that *In1.1C* down-regulates *RANTES* transcriptional activity.

By contrast, there was no difference in transcriptional activity between haplotypes *GCT* and *ACT*, or between *GCC* and *ACC*, demonstrating no obvious effect on transcription by *−403A*. A moderate up-regulating role of *−28G* was observed in the comparison of *AGC* to *ACC* (78% increase, $P = 0.006$) but not of *AGT* to *ACT*. These experiments demonstrate a strong down-regulation of *RANTES* transcription by *In1.1C* and a modest up-regulation transcription action of *−28G* (Fig. 1*d*).

Effect of *RANTES* SNPs and Haplotypes on HIV-1 Infection. We compared the frequencies of the *RANTES* variant alleles between high-risk HIV-1-exposed uninfected (HREU) individuals and HIV-1-infected seroconverters for EA and AA. The variant alleles, *−403A*, *In1.1C*, and *3′222C* each showed a diminished frequency in the HREU group [odds ratio (OR) = 1.45, 1.41, and 1.53; $P = 0.06$, 0.11, and 0.08, respectively; Fig. 2, Table 2, which is published as supporting information on the PNAS web site, www.pnas.org], suggesting an association with increased susceptibility to HIV-1 infection. For combined ethnic groups the *−403A*, *In1.1C*, and *3′222C* variant alleles showed significant association with increased risk of HIV-1 infection (OR = 1.44, 1.41, and 1.51; $P = 0.02$, 0.04, and 0.04, respectively, Fig. 2, Table 2). The haplotypes R2–R5 carrying the variant alleles were all nonsignificantly elevated in the HIV-1-infected study participants. The positive linkage disequilibrium between the four variants precludes implication of a single associated variant SNP with HIV-1 infection, but lower *RANTES* levels specified by *In1.1C* poses it is an attractive candidate.

Effect of *RANTES* SNPs and Haplotypes on AIDS Progression. The influences of individual *RANTES* SNP alleles and multisite haplotypes on AIDS progression were evaluated for dominant, codominant, and recessive genetic models among EA ($n = 673$) and AA ($n = 291$) HIV-1 seroconverters by using a Cox proportional hazards model (41). Three AIDS endpoints reflecting advancing morbidity were evaluated: (*i*) CD4 < 200 cells per mm^3, (*ii*) AIDS-1987, as defined by the Centers for Disease Control and Prevention (38); and (*iii*) AIDS-related death. Analyses with and without adjustments for the influence of described AIDS-restriction gene covariates are listed in Table 1 and illustrated in Fig. 3.

A strong SNP genotypic association in the Cox model analysis involved AA seroconverters carrying one or two copies of the *In1.1C*. This allele conferred more rapid progression to AIDS-1987 [relative hazard (RH) = 1.9, $P = 0.009$] or death [RH = 2.5, $P = 0.008$ and RH = 4.6, $P = 0.002$ for dominant and recessive (not shown) models, respectively] (Fig. 3*b*, Table 1). The EA cohorts did not show the *In1.1C* influence when tested separately (Fig. 3*a*, Table 1; but see below). No significant associations for rapid AIDS progression were observed for *−403A*, *−28G*, or *3′222C*-bearing

Table 1. Survival analysis of *RANTES* allele-bearing genotypes and haplotype-bearing genotypes for association with progression to AIDS endpoints by using the Cox proportional hazard model (dominant model)

			−403A			*−28G* (=R5)†			*In1.1C*			*3′222C* (≈R4)‡			R2			R3			R3 + R5§		
	Outcome	*N*	RH	(95% CI)	*P*	RH	(95% CI)	*P*	RH	(95% CI)	*P*	RH	(95% CI)	*P*	RH	(95% CI)	*P*	RH	(95% CI)	*P*	RH	(95% CI)	*P*
Adjusted*																							
EA	CD4 < 200	659	0.86	(0.69, 1.07)	0.17	0.93	(0.58, 1.49)	0.76	0.85	(0.67, 1.08)	0.19	0.85	(0.66, 1.1)	0.22	1.01	(0.71, 1.46)	0.94	4.64	(1.7, 12.63)	0.003	1.06	(0.69, 1.63)	0.79
	AIDS-1987	672	1.14	(0.9, 1.44)	0.27	1.24	(0.78, 1.99)	0.36	1.06	(0.83, 1.36)	0.65	0.96	(0.73, 1.27)	0.78	1.20	(0.82, 1.76)	0.35	2.10	(0.67, 6.6)	0.21	1.36	(0.88, 2.1)	0.16
	Death	671	1.13	(0.88, 1.45)	0.33	1.20	(0.72, 2.03)	0.48	1.16	(0.89, 1.51)	0.27	1.10	(0.83, 1.47)	0.51	1.03	(0.67, 1.56)	0.91	1.99	(0.63, 6.29)	0.24	1.29	(0.8, 2.07)	0.29
AA	CD4 < 200	287	0.77	(0.51, 1.15)	0.20	N/A			1.09	(0.74, 1.62)	0.66	1.14	(0.66, 1.96)	0.64	0.75	(0.51, 1.12)	0.16	1.01	(0.64, 1.58)	0.98	1.01	(0.64, 1.58)	0.98
	AIDS-1987	291	1.47	(0.82, 2.64)	0.19	N/A			1.93	(1.18, 3.18)	0.009	1.52	(0.8, 2.88)	0.20	0.94	(0.58, 1.55)	0.82	1.70	(1.01, 2.87)	0.05	1.70	(1.01, 2.87)	0.05
	Death	291	1.39	(0.63, 3.07)	0.41	N/A			2.50	(1.28, 4.91)	0.008	1.89	(0.85, 4.21)	0.12	0.70	(0.35, 1.4)	0.31	2.30	(1.16, 4.54)	0.02	2.30	(1.16, 4.54)	0.02
Combined	CD4 < 200	945	0.83	(0.69, 1.01)	0.07	0.94	(0.59, 1.5)	0.80	0.91	(0.74, 1.11)	0.34	0.88	(0.7, 1.11)	0.29	0.88	(0.67, 1.15)	0.35	1.18	(0.77, 1.8)	0.44	1.05	(0.77, 1.42)	0.78
	AIDS-1987	962	1.19	(0.96, 1.47)	0.11	1.27	(0.8, 2.03)	0.31	1.20	(0.96, 1.49)	0.11	1.02	(0.8, 1.32)	0.85	1.10	(0.81, 1.49)	0.55	1.74	(1.08, 2.81)	0.02	1.49	(1.08, 2.07)	0.02
	Death	961	1.16	(0.92, 1.47)	0.21	1.23	(0.73, 2.06)	0.44	1.29	(1.01, 1.64)	0.04	1.16	(0.89, 1.52)	0.28	0.93	(0.64, 1.34)	0.70	2.18	(1.23, 3.88)	0.008	1.54	(1.06, 2.25)	0.02
Unadjusted																							
EA	CD4 < 200	659	0.90	(0.72, 1.12)	0.34	1.19	(0.75, 1.89)	0.46	0.92	(0.73, 1.17)	0.51	0.86	(0.67, 1.11)	0.25	0.95	(0.66, 1.36)	0.78	4.77	(1.75, 12.98)	0.002	1.33	(0.87, 2.03)	0.18
	AIDS-1987	672	1.20	(0.96, 1.52)	0.12	1.73	(1.09, 2.74)	0.02	1.18	(0.92, 1.52)	0.18	0.99	(0.76, 1.31)	0.97	1.07	(0.73, 1.56)	0.74	2.23	(0.71, 6.98)	0.17	1.79	(1.17, 2.74)	0.008
	Death	671	1.20	(0.94, 1.54)	0.15	1.51	(0.9, 2.51)	0.12	1.26	(0.97, 1.64)	0.09	1.12	(0.84, 1.49)	0.43	1.00	(0.65, 1.52)	0.98	2.20	(0.7, 6.91)	0.18	1.57	(0.98, 2.51)	0.06
AA	CD4 < 200	287	0.79	(0.53, 1.18)	0.26	N/A			1.08	(0.73, 1.59)	0.71	1.06	(0.62, 1.8)	0.84	0.75	(0.51, 1.12)	0.16	1.02	(0.66, 1.59)	0.92	1.02	(0.66, 1.59)	0.92
	AIDS-1987	291	1.44	(0.81, 2.57)	0.22	N/A			1.78	(1.09, 2.91)	0.02	1.46	(0.77, 2.76)	0.25	0.94	(0.57, 1.55)	0.81	1.57	(0.94, 2.63)	0.08	1.57	(0.94, 2.63)	0.08
	Death	291	1.41	(0.64, 3.1)	0.39	N/A			2.31	(1.19, 4.5)	0.01	1.85	(0.83, 4.11)	0.13	0.73	(0.37, 1.44)	0.37	2.07	(1.07, 4.03)	0.03	2.07	(1.07, 4.03)	0.03
Combined	CD4 < 200	945	0.87	(0.72, 1.06)	0.17	1.19	(0.75, 1.89)	0.46	0.96	(0.79, 1.18)	0.70	0.89	(0.71, 1.13)	0.34	0.85	(0.65, 1.12)	0.24	1.18	(0.78, 1.8)	0.43	1.17	(0.86, 1.59)	0.32
	AIDS-1987	962	1.23	(1, 1.53)	0.05	1.73	(1.09, 2.74)	0.02	1.29	(1.03, 1.6)	0.02	1.05	(0.82, 1.35)	0.71	1.02	(0.75, 1.38)	0.91	1.66	(1.03, 2.67)	0.04	1.70	(1.22, 2.36)	0.002
	Death	961	1.22	(0.96, 1.55)	0.10	1.51	(0.9, 2.51)	0.12	1.37	(1.07, 1.74)	0.01	1.18	(0.9, 1.54)	0.23	0.91	(0.63, 1.31)	0.61	2.10	(1.18, 3.75)	0.01	1.71	(1.18, 2.5)	0.005

CI, confidence interval; RH, relative hazard; N/A, not available. *, Adjusted with covariates (see *Materials and Methods*). †, *−28G* is equivalent to haplotype R5. ‡, *3′222C* is virtually equivalent to haplotype R4 (99.7% of EA and all AA haplotypes carrying *3′222C* are R4. §, Results for haplotypes R3 and R5 combined.

genotypes, in analyses adjusted for known AIDS restriction genes (Table 1).

To clarify the association of the individual SNPs, we next analyzed the influence of composite SNP haplotypes R1–R5 (Fig. 1*a*) on the rate of progression to AIDS. Evaluation of haplotype association with the AIDS progression also implicated the *In1.1T/C* site as responsible for the observed allele association (Table 1, Fig. 3 *c–f*). The R3 haplotype (which contains *In1.1C*) showed significant AIDS accelerating influence in AA (RH = 1.7, $P = 0.05$, for AIDS-1987 and RH = 2.3, $P = 0.02$ for AIDS-related death), in EA (RH = 4.6, $P = 0.003$ for CD4 < 200), and in combined cohorts (RH = 1.7, $P = 0.02$ for AIDS-1987; RH = 2.2; $P = 0.008$ for AIDS-related death). A weaker association for the *In1.1C*-containing R4 haplotype carriers with rapid AIDS progression was evident in AA (Fig. 3 *d* and *f*) but not in EA (*c* and *e*).

The strong AIDS accelerating influence of *In1.1C* in AA but not in EA apparently derives from the difference in haplotype frequencies between the two groups: the strongly accelerating haplotype R3 represents 64% of *In1.1C*-carrying haplotypes for AA, but only 3% of *In1.1C* haplotypes for EA. The EA R5 haplotype, (which is infrequent in AA), carries both *In1.1C* and *−28G*, regulating alleles that influence transcription in opposite directions (Fig. 1*d*). The counteracting influence of *In1.1C* and *−28G* is illustrated epidemiologically in an AIDS survival analysis of EA that compares R5 to R3 haplotypes (Fig. 3 *c* and *e*). R5 haplotypes that retain the offsetting *In1.1C* and *−28G* alleles only slightly accelerate AIDS, whereas R3 haplotype with *In1.1C* and the wild-type *−28C* allele show a much stronger influence toward rapid AIDS progression.

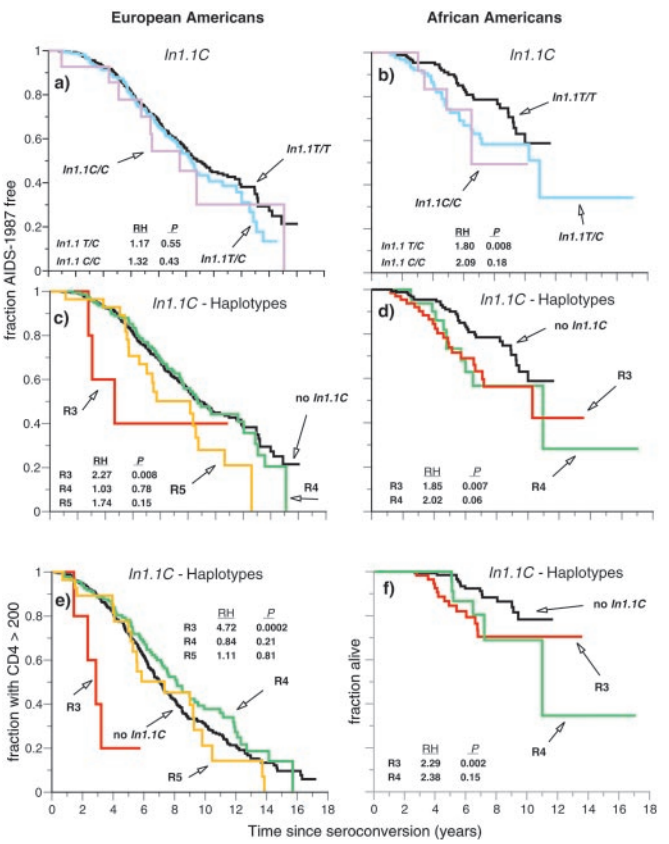

Fig. 3. Kaplan–Meier survival curves for progression to AIDS-1987 (*a–d*), CD4 < 200, AIDS related death comparing the influence of the *RANTES In1.1C* allele, and *In1.1C*-carrying haplotypes on progression to AIDS by HIV-1-infected EA (*Left*) and AA (*Right*). Cox model RH and Kaplan–Meier Wilcoxon *P* values comparing each variant genotype or haplotype to the no-*In1.1C* group are shown for each factor. (*a* and *b*) Rate of progression to AIDS-1987 based on *In1.1C* genotype: black, no *In1.1C*; blue, one copy; and purple, two copies of *In1.1C* for EA (*a*) and AA (*b*). (*c–f*) Rate of progression to AIDS partitioned according to *In1.1C*-containing haplotypes: black, no *In1.1C*; red, R3; green, R4; and orange, R5. (*c* and *e*) Progression of EA to AIDS-1987 (*c*) and CD4 < 200 (*e*). (*d* and *f*) Progression of AA to AIDS-1987 (*d*) and AIDS-related death (*f*). Subjects carrying two different *In1.1C* haplotypes (two EA and six AA) are omitted.

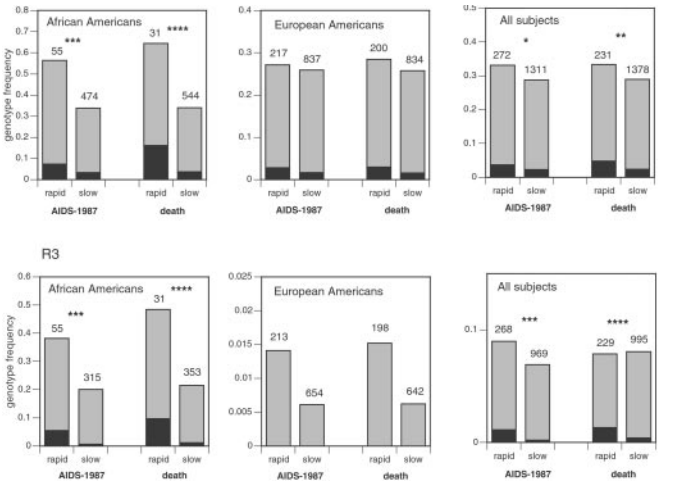

Fig. 4. Defined disease category analysis of *RANTES In1.1C* and the *In1.1C*-carrying R3 haplotype comparing slow versus rapid progressors to AIDS-1987 and AIDS-related death. Bars show frequencies of homozygotes (black) and heterozygotes (gray) for *In1.1C* or the R3 haplotype in the slow and rapid groups for AA (*Left*), EA (*Center*), and combined ethnic groups (*Right*). Number of subjects considered and significance for a codominant model by Fisher's exact test are indicated (*, $P < 0.05$; **; $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

The detrimental effects of *In1.1C* on HIV-1-infected individuals were also apparent in categorical analyses of slow versus rapid progressors to AIDS. This approach allows the inclusion of seroprevalent individuals (those whose seroconversion date is unknown because they were HIV-1 antibody-positive at the time of study enrollment) in the slow/nonprogressor category (26). An elevation in *In1.1C* allele frequency was observed among the rapid progressor groups for AIDS-1987 and AIDS-death in both dominant and codominant (Fig. 4) models. The association was highly significant in AA for AIDS-1987 and death (OR = 2.54, $P = 0.002$ and OR = 3.53, $P = 0.0009$, respectively, Fisher's exact test for the dominant model). An association for the R3 haplotype was also apparent (Fig. 4) and was highly significant both for AA (OR = 2.47, 3.42; $P = 0.005$, 0.002) and for combined EA and AA (OR = 2.45, 3.20; $P = 0.002$, 0.0004), respectively, for AIDS-1987 and AIDS-related death, dominant model. The Mantel–Haenszel trend test showed stronger associations for the codominant than for the dominant model for almost all cases, suggesting a gene dose effect. These survival and categorical results strongly implicate a role for the intronic regulatory allele *In1.1C* polymorphism in promoting the rate of AIDS progression.

A quantitative estimate for the *In1.1C*-bearing haplotypes on AIDS progression in the study population can be determined by computing the attributable fraction, a parameter that combines the strength of the epidemiological influence (relative risk) and the frequency of the protective genotype (40). To estimate the attributable fraction of *In1.1C* for progression to AIDS-related death, relative risk was obtained from a categorical analysis with AA seroconverters. The calculated relative risk (2.61, confidence interval 1.38–4.95) combined with the *In1.1C* allele frequency (36%) in AA indicate that 37% (confidence interval: 12–59%) of rapid (within 7.5 years) progression to clinical AIDS can be attributed to the detrimental effect of the *RANTES In1.1C* allele.

Discussion

We describe here the validation of seven SNPs within the *RANTES* gene and analyze the four common variants

(*−403G/A*, *−28C/G*, *In1.1T/C*, and *3′222T/C*) for genetic association with HIV-1 transmission, AIDS progression, and gene transcription. *In1.1T/C* is shown to differentially bind nuclear proteins (Fig. 1*b*) and have powerful regulatory activity on gene expression (Fig. 1 *c* and *d*). The *In1.1C* allele results in reduced *RANTES* transcription and is associated with more rapid progression to AIDS (Figs. 3 and 4, Table 1). The rapid disease course of an estimated 37% of AA AIDS patients whose disease progresses within 7.5 years after HIV-1 infection can be attributed to their *RANTES–In1.1C*-bearing genotype. Because 36% of AA carry the *In1.1C* allele, it is likely that *In1.1C* may have a significant impact on the AIDS epidemic in sub-Saharan Africa.

The results suggested that *In1.1C* also increases susceptibility to HIV-1 infection and accelerates progression to AIDS in EA, but these effects are confounded and partially quenched by the nearly total positive linkage disequilibrium between *In1.1C* and the other *RANTES* variants studied. Three common *RANTES* variants and their inclusive haplotype R4 were associated with increased risk of infection (Fig. 2). That association plus the demonstrated down-regulation of *RANTES* transcription by *In1.1C* (Fig. 1 *b–d*) implicate *In1.1C* as regulating in the HIV-1 infection process. *In1.1C* shows little influence on AIDS progression in EA (Fig. 3*a*); however, the R3 haplotype, carrying *In1.1C*, accelerates AIDS progression in both EA and AA (Table 1, Fig. 3 *c–f*). In EA this haplotype is rare, and its effect is diluted by the effects of the R5 haplotype, which carries the *−28G* variant that we show to counter the down-regulating effect of *In1.1C*, and the R4 haplotype, in which the effect of *In1.1C* appears to be countered by unknown factors. The haplotype analysis, along with the demonstrated down-regulating role of the *In1.1C* allele on *RANTES* transcription, strongly implicates *In1.1C* as a highly associated genetic risk factor for progression to AIDS and AIDS-related death. The R2 haplotype (carrying *−403A* but lacking *In1.1C*) has no accelerating effect (RH < 1.15, $P > 0.4$, Table 1), arguing against *−403A* as a causative factor (9, 22).

Because CCR5 ligands have been shown to competitively bind to and reduce surface expression of CCR5, down-regulation of *RANTES* may increase the number of binding targets for HIV-1, thus promoting HIV-1 replication (1, 10–20). The susceptible influence of *In1.1C* is likely caused by an *in vivo* reduction in RANTES–CCR5 binding, which may increase HIV-1 replication and rate of progression to AIDS. The association of the down-regulating variant of *RANTES* with accelerated progression to AIDS is clinically relevant because modified forms of RANTES are under active investigation as HIV-1-antiviral agents (41). The *in vitro* and *in vivo* evidence in this study argues for a beneficial role of high RANTES levels in limiting progression to AIDS in HIV-1-infected individuals. Our results would predict that RANTES levels would be diminished in individuals with an *In1.1C*-bearing genotype.

The consistent genetic association shown here between susceptibility to HIV-1 transmission and disease progression by a physiologically plausible down-regulating *In1.1C* variant clarifies the underlying complex mechanisms of *RANTES* genetic influence on HIV-1 pathogenesis and provides valuable information in evaluating RANTES for therapeutic purposes.

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