CCR5 promoter human haplogroups associated with HIV-1 disease progression in Thai injection drug users

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Background: An evolutionary-based analysis of the CC chemokine receptor 5 gene (*CCR5*) promoter region has identified nine stable human haplogroups, within which certain haplogroups appear to influence HIV-1 disease progression differentially among Caucasians and African-Americans.

Objective: To assess the influence of *CCR5* haplogroups on HIV-1 disease progression in a Thai population.

Design: Haplogroup analysis of HIV-1-seropositive injection drug users (IDU) participating in a prospective cohort study in Bangkok. All were documented seroconverters with a median follow-up time of 3.5 years (range, 0.2–7.0).

Methods: From a cohort of 130 IDU, 106 (81.5%) were genotyped for the *CCR2b-64I, CCR5-Δ32* and seven *CCR5* promoter alleles constituting the *CCR5* haplogroups. Survival curves and adjusted Cox proportional hazards models were used to assess the effect of haplogroups on the time from HIV-1 infection until CD4 count $< 200 \times 10^6$ cells/l.

Results: The most common *CCR5* haplogroups were HHC (61.8%), followed by HHE (15.6%) and HHF*2 (14.6%). HHE was associated with an accelerated CD4 count decline to $< 200 \times 10^6$ cells/l (adjusted relative hazard, 1.88; 95% confidence interval, 1.05–3.36; $P = 0.02$).

Conclusions: This is the first evidence that the *CCR5* haplogroup E speeds the decline of the CD4 cell count and may lead to accelerated disease progression among HIV-infected Thais. These new observations highlight the need for additional studies involving populations in Asia.

AIDS 2004, **18**:1327–1333

Keywords: Asia, HIV-1, *CCR5*, *CCR2*, host genetics

Introduction

The CC chemokine receptor 5 gene (*CCR5*) encodes a cell surface receptor that serves as the principal coreceptor for HIV-1 entry into CD4 T lymphocytes. Since this pivotal discovery, *CCR5* has become a focus of host genetic studies evaluating HIV-1 pathogenesis [1,2]. Indeed, the role of CCR5 in pathogenesis was first

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Received: 26 January 2004; revised: 19 March 2004; accepted: 20 April 2004.

DOI: 10.1097/01.aids.0000131303.39957.15

ISSN 0269-9370 © 2004 Lippincott Williams & Wilkins

highlighted in a study demonstrating that persons homozygous for a 32 base pair (bp) deletion in the open reading frame of *CCR5* (*CCR5-Δ32*) were relatively resistant to HIV-1 infection [3]. Polymorphisms in *CCR5* regulatory or promoter region may also influence cell surface expression and, consequently, influence susceptibility to infection by HIV-1 [4–6]. Although multiple polymorphisms throughout *CCR5* have been examined, most appear to exist at low frequencies and are often distributed in different proportions in different populations [1,7]. For instance, the best-characterized *CCR5* variant, the *CCR5-Δ32* allele, is common among persons of European descent but is virtually absent in African-Americans, Africans and Asians [8,9].

The effect of *CCR2b*, another chemokine receptor gene closely linked to *CCR5*, on HIV-1 disease progression has also been studied. The *CCR2b-64I* allele has been associated with delayed progression to AIDS [10–12] and, unlike *CCR5* polymorphisms, is relatively common among major ethnic groups [13]. However, it remains unclear how a variant for a chemokine receptor that most strains of HIV-1 do not use for cell entry can affect disease progression. Proposed mechanisms include a time-dependent interaction with CCR5 [14] or a differential interaction with CXCR4 [15,16]. Another possible explanation is that *CCR2b-64I* is tracking another mutation through linkage disequilibrium [10], which has been documented between *CCR2b-64I* and certain *CCR5* promoter mutations [1,11]. These findings led to investigations of *CCR2b-CCR5* haplogroups [13] and a recent classification of these alleles into nine evolutionarily distinct *CCR5* human haplogroups designated as HHA to HHE, HHF*1, HHF*2, HHG*1 and HHG*2 [17]. The haplogroups are characterized by a collection of unique polymorphisms at distinct positions in the *CCR5* promoter, *CCR5-Δ32* and *CCR2b-64I* [13,17].

Certain *CCR5* haplogroups have been shown to influence HIV-1 transmission and disease progression differently according to their distribution by ethnic group [13,18], suggesting a strong interaction between haplogroup and ethnic population. In addition, several studies have demonstrated that disease acceleration or retardation associated with specific haplogroups differs between African-Americans and Caucasians [13,19]. Despite these intriguing findings, data are scarce regarding the distribution and relative effects of *CCR5* haplogroups on HIV-1 pathogenesis among Asian populations. In Thailand, the HIV-1 epidemic was characterized by the independent introduction and spread of two different HIV-1 subtypes, B and CRF01_AE [20–22]. A cohort of seroconverting injection drug users (IDU) followed prospectively in Bangkok [23,24] has provided a unique opportunity to address the effect of *CCR5* haplogroups on HIV-1 disease progression in an Asian population.

Methods

Study population

The Bangkok Metropolitan Administration (BMA) manages a large municipal drug treatment program in Thailand where approximately 8000–10 000 drug users are seen annually. As described previously, 130 seroconverters were identified between 1995 and 1998 from a prospective cohort of over 1200 IDU [23,24]. Study protocols were approved by the Ethical Research Committee, Ministry of Public Health (Nonthaburi, Thailand) and the Institutional Review Board, Centers for Disease Control and Prevention (Atlanta, Georgia, USA). After voluntary informed consent was obtained, blood samples were collected after the first HIV-1-seropositive visit, 1 month later and then at intervals of 4 months. The estimated date of seroconversion was defined as the midpoint between the dates of the last negative enzyme immunoassay (EIA) and the first positive EIA. Median time between the last negative EIA and estimated seroconversion was 2 months. Previous characterization of infecting viruses showed that 103 (79.2%) of the IDU were infected with HIV-1 CRF01_AE strains, and 27 (20.8%) were infected with HIV-1 subtype B strains [25]. The median age at estimated seroconversion was 31 years (range, 19–50), and most of the subjects were male (89.2%). Very little antiretroviral therapy was used in Thailand at the time of the observations, especially early in the course of HIV-1 infection. The analysis of disease progression only applied to the period before the study subjects began antiretroviral therapy.

HIV-1 viral load and CD4 T lymphocyte determination

Peripheral blood mononuclear cells (PBMC) were frozen in liquid nitrogen, and all cell pellets and plasma samples were frozen at −70°C within 8 h of collection. Plasma HIV-1 RNA viral load was quantified by the Amplicor HIV-1 Monitor Test version 1.5 (Roche Diagnostics, Branchburg, New Jersey, USA). CD4 T lymphocyte counts were determined using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, California, USA).

Genotyping

All the genotyping analyses were performed by individuals with no knowledge of the clinical information on the study participants. Genomic DNA was extracted from the PBMC of all participants using commercial kits, according to the manufacturer's protocols (Qiagen, Chatsworth, California, USA). Among the 130 IDU, 106 (81.5%) were genotyped for polymorphisms in the *CCR2b-64I, CCR5-Δ32* and *CCR5* promoter regions. The primers and polymerase chain reaction (PCR) conditions have been described previously for *CCR5-Δ32* [26] and *CCR2b-64I* [18] genotyping. In brief, target sequences were amplified by PCR from genomic

DNA. For the *CCR5-Δ32* mutation, PCR products were separated by agarose gel electrophoresis, and two fragments of 198 bp and 166 bp were obtained that corresponded to the normal and the 32 bp deletion alleles. For the *CCR2b-64I* mutation, the PCR products were digested with *Bsa*BI (New England BioLabs, Beverly, Massachusetts, USA), and the alleles were identified by agarose gel separation of the resulting fragments.

Polymorphisms in the *CCR5* promoter region were determined by PCR amplification of the target fragments, followed by direct sequencing of the PCR products. Detailed information for primers, PCR conditions, and sequencing has been given elsewhere [27]. Briefly, the promoter region of *CCR5* was amplified by PCR from genomic DNA. The purified PCR products were sequenced bidirectionally using the BigDye terminator cycle-sequencing ready reaction kit, following the manufacturer's protocol, and then run on an ABI 377 DNA sequencer (PE Applied Biosystems, Foster City, California, USA). The sequences were edited, and mutations were identified only when sequencing peaks in both strands were visible. *CCR5* haplogroups were assigned to each specimen on the basis of the constellation of unique point mutations in the *CCR2* and the *CCR5* genes, as described previously [13,17].

Statistical analysis

Kaplan–Meier survival curves were used to evaluate the predictive value of the *CCR5* haplogroups for the time from estimated HIV-1 infection until the CD4 T lymphocyte count declined to $< 200 \times 10^6$ cells/l. Although no antiretroviral therapy was reported for any of the seroconverters during the first 12 months following seroconversion, antiretroviral therapy for persons infected with HIV increased substantially in Thailand during the latter period of this study. In October 2001, the BMA made treatment guidelines such that three antiretroviral drugs and prophylaxis for opportunistic infections were offered when HIV-1-infected individuals had CD4 cell counts $< 200 \times 10^6$ cells/l. Therefore, all data observations up to the first reported antiretroviral therapy were right-censored so that analyses and comparisons reported in this study came from data contributed only by seroconverters when they were treatment naive. The analyses were repeated with all the data points without right-censoring to evaluate the effects of antiretroviral therapy on the natural history of CD4 cell counts. Between-group comparisons were based upon the log-rank test. Relative hazards (RH) and corresponding 95% confidence intervals (CI) were calculated using Cox proportional hazards models adjusted for age at estimated seroconversion. Proportionality of the hazards in various groups were examined graphically and/or tested in a Cox model that included an interaction term between time and *CCR5* haplogroup. Hardy–Weinberg analysis was performed on the genotype distribution data. Analyses were completed using the SAS System for Windows, version 8.2 (SAS Institute, Cary, North Carolina, USA).

Results

Distribution of alleles and haplogroups

CCR2b, CCR5-Δ32 and *CCR5* promoter allele and genotype frequencies are reported in Table 1. Although only a limited number of individuals were genotyped for the mutations, the overall genotype distributions followed Hardy–Weinberg equilibrium ($P > 0.05$). None of the IDU carried the *CCR5-Δ32* mutation. The *CCR2b-64I* allele occurred at a frequency of 14.6%, and nearly all persons carrying the allele were heterozygotes. The most common *CCR5* promoter alleles were *CCR5-2554T* and *CCR5-2086G* (61.8%), followed by *CCR5-2459A* and *CCR5-2135C* (32.1%). Interestingly, the distribution of *CCR5-2554T* and *CCR5-2086G* differed by infecting viral strains ($P < 0.001$). IDU infected with CRF01_AE (111/172 alleles, 65%) were more likely to carry either of these alleles than those infected with subtype B (20/40 alleles, 50%).

In this cohort, the most common human *CCR5* haplogroup was HHC (61.8%), followed by HHE

Table 1. Frequencies of alleles and genotypes of *CCR2b, CCR5-Δ32* and *CCR5* promoter region.

Allele	No. (%)	Genotype	No. (%)
CCR2b			
64V	181 (85.4)	V/V	76 (71.7)
64I	31 (14.6)	V/I	29 (27.4)
CCR5-Δ32		I/I	1 (0.9)
Wild-type	212 (100.0)	+/+	106 (100.0)
Deletion	0 (0.0)	+/–	0 (0.0)
CCR5P		–/–	0 (0.0)
–2733A	209 (98.6)	A/A	103 (97.2)
–2733G	3 (1.4)	A/G	3 (2.8)
		G/G	0 (0.0)
–2554G	81 (38.2)	G/G	13 (12.3)
–2554T	131 (61.8)	G/T	55 (51.9)
		T/T	38 (35.8)
–2459G	144 (67.9)	G/G	45 (42.5)
–2459A	68 (32.1)	G/A	54 (50.9)
		A/A	7 (6.6)
–2135T	144 (67.9)	T/T	45 (42.5)
–2135C	68 (32.1)	T/C	54 (50.9)
		C/C	7 (6.6)
–2132C	212 (100.0)	C/C	106 (100.0)
–2132T	0 (0.0)	C/T	0 (0.0)
		T/T	0 (0.0)
–2086A	81 (38.2)	A/A	13 (12.3)
–2086G	131 (61.8)	A/G	55 (51.9)
		G/G	38 (35.8)
–1835C	180 (84.9)	C/C	75 (70.8)
–1835T	32 (15.1)	C/T	30 (28.3)
		T/T	1 (0.9)

(15.6%) and HHF⋆2 (14.6%). The three minor haplogroups were HHA (6.1%), HHF⋆1 (0.5%) and HHG⋆1 (1.4%). None of these IDU carried HHB, HHD and HHG⋆2, the *CCR5*-Δ*32*-bearing haplogroup. The frequency of *CCR5* haplogroup genotypes is reported in Table 2.

Survival analysis of the CCR5 haplogroups

Survival analyses were conducted using the carrier status (1 or 2 versus 0 copies) of each haplogroup.

Table 2. Frequency of *CCR5* haplogroup genotypes.

Genotype	No. (%)
A/C	7 (6.6)
A/E	3 (2.8)
A/F*1	1 (0.9)
A/F*2	1 (0.9)
A/G*1	1 (0.9)
C/C	38 (35.9)
C/E	19 (17.9)
C/F*2	28 (26.4)
C/G*1	1 (0.9)
E/E	5 (4.7)
E/G*1	1 (0.9)
F*2/F*2	1 (0.9)

Survival curves for the four most prevalent haplogroups, HHA, HHC, HHE and HHF⋆2, are shown in Fig. 1. To evaluate other markers of disease progression, longitudinal plots of log-transformed HIV-1 viral load (Fig. 2) and CD4 T lymphocyte count (Fig. 3) were also examined for these haplogroups.

The survival curve for HHC appears to indicate that a slower disease progression occurred later during the course of HIV-1 infection (Fig. 1B). Further analysis also indicated a dose–response effect for HHC when IDU were categorized into groups having two, one or no HHC haplogroups (data not shown). The high frequency of HHC allowed further investigation of HHC-bearing genotypes, but no significant modification to survival was observed.

There was a significant association between HHE and rapid CD4 T lymphocyte count decline to $< 200 \times 10^6$ cells/l in this Thai cohort ($P = 0.02$; Fig. 1C). HHE conveyed a nearly two-fold increased relative risk of rapid CD4 cell count decline (adjusted RH, 1.88; 95% CI, 1.05–3.36). When HHE carriers were categorized in a dose–response manner, a trend towards

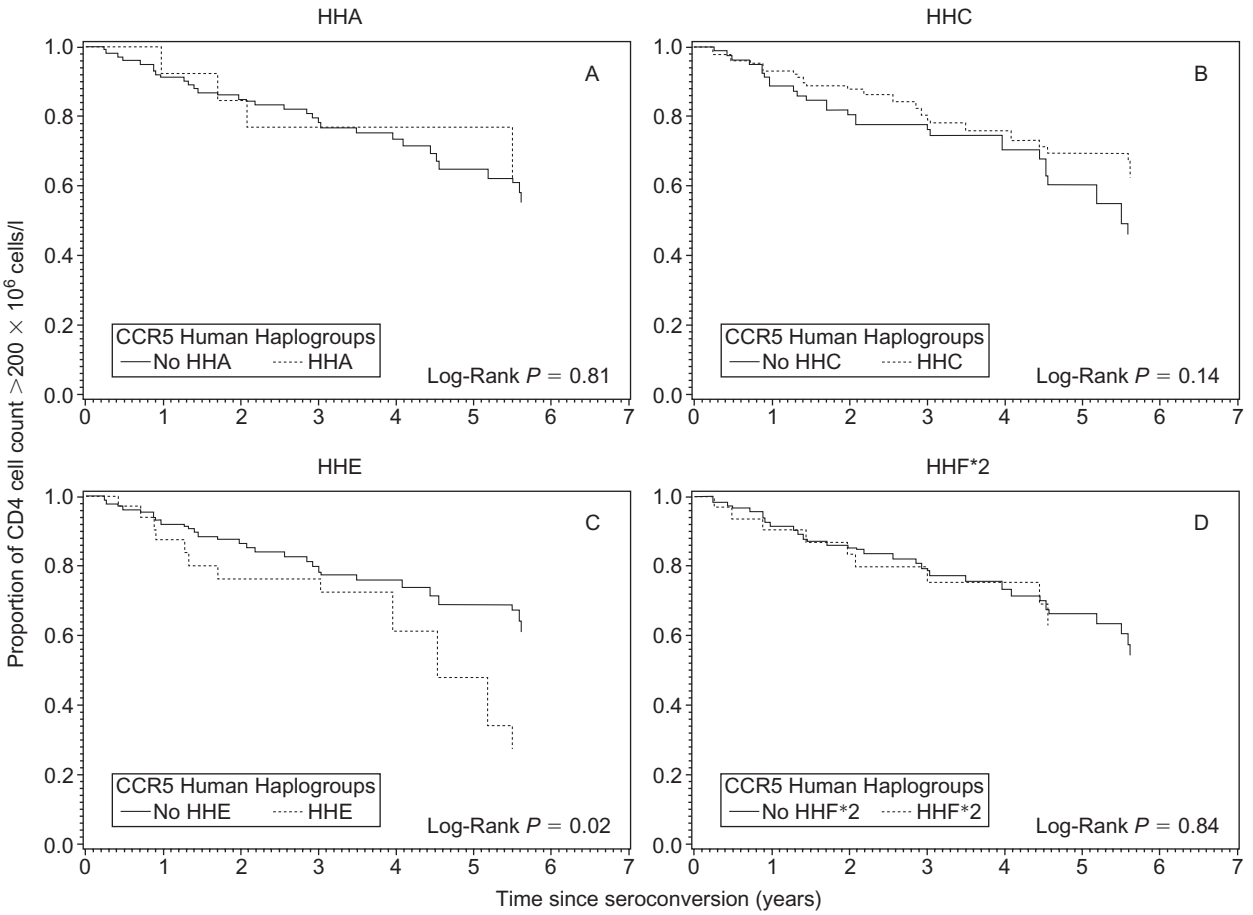

Fig. 1. Kaplan–Meier survival curves from the time of estimated initial HIV-1 infection to CD4 T lymphocytes decline to < 200 × 10^6cells/l, according to the *CCR5* human haplogroup (HH).

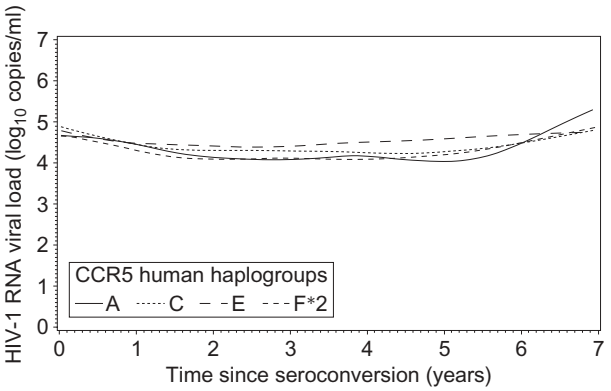

Fig. 2. Longitudinal plots of HIV-1 viral load, according to *CCR5* human haplogroup.

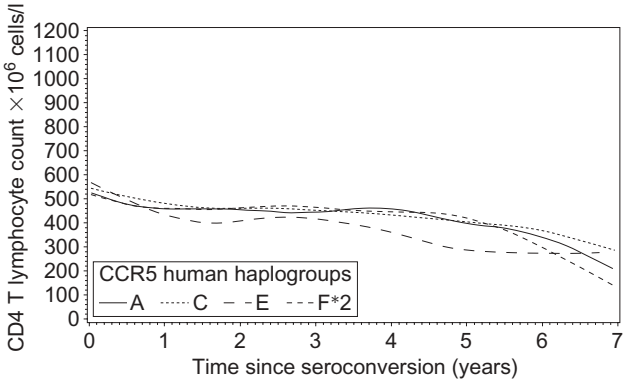

Fig. 3. Longitudinal plots of CD4 T lymphocyte count, according to *CCR5* human haplogroup.

differential disease progression could be seen among those with two, one or no HHE haplogroups ($P = 0.11$; data not shown). Compared with the other haplogroups, HHE carriers appeared to have higher viral loads (Fig. 2) and lower CD4 cell counts during follow-up (Fig. 3). When all follow-up data were used for the analysis, CD4 cell count decline in HHE carriers was no longer significant compared with non-HHE carriers, and the survival curves for HHA, HHC and HHE were crossover toward the end of the follow-up, indicating that antiretroviral therapy had altered the natural histories of CD4 T cell count decline in these IDU (data not shown).

A protective effect was not observed for the *CCR2b-64I*-bearing haplogroup HHF⋆2 (Fig. 1D). Examination of the *64I* allele as a predictor for disease progression confirmed this lack of association, as did another analysis controlling for the detrimental effect of HHE (data not shown). Consistent with a lack of protective effect, no indication was evident of a lower viral load level among persons with an HHF⋆2 haplogroup (Fig. 2). In fact, HHF⋆2 carriers appeared to have the lowest CD4 cell counts by the end of follow-up (Fig. 3).

Discussion

To date, knowledge about the effects of *CCR5* haplogroups on HIV-1 infection and disease progression has been based primarily on Caucasian and African-American populations. Our study provides the first evidence that carrying the HHE haplogroup is associated with more rapid disease progression among Asian populations. This finding is consistent with observations among a number of Caucasian and African-American cohorts [4,13,19,28] and with a study demonstrating the influence of HHE on perinatal HIV-1 transmission [29]. A recent study of Caucasian homosexual men also found that HHE was more prevalent among rapid progressors than slow progressors. In addition, HHE homozygotes experienced a more rapid decline of CD4 T lymphocytes than persons not carrying this haplogroup (C. Yang *et al.*, unpublished data). Taken together, these findings suggest that HHE may have a significant impact on clinical HIV-1 disease progression because of its role in promoting HIV-1 pathogenesis in diverse populations.

Our study did not observe a protective effect against HIV-1 disease progression for the HHF⋆2 haplogroup among Thai participants. This was not surprising because early studies of the *CCR2b-64I* allele, for which the HHF⋆2 haplogroup is characterized, have been conflicting. They either demonstrated an association with slower disease progression among Caucasians but not African-Americans [10,30] or among African-Americans but not Caucasians [18,31]. Given that allele frequencies may vary widely within and between populations irrespective of disease status [32], the presence of other modulating genes or unidentified polymorphisms that differ by ethnic group may contribute to observed differences in association through linkage disequilibrium. Alternatively, the protection conferred by HHF⋆2 may be related to initial acquisition of HIV-1 rather than progression, as observed by differences in viral load over time [28] as well as in studies of perinatal exposure [29], discordant couples in Thailand [33] and highly exposed persistently HIV-1-seronegative women [27]. However, the smaller sample size in the current study may also contribute to the insignificant result on the protective effect of *CCR2b-64I* on HIV-1 disease progression. Studies with larger sample sizes are needed to address this issue.

It appears that individuals carrying HHC had slower disease progression during the late course of HIV-1 infection, at 4 years after seroconversion. Studies with a larger cohort are needed to explore this finding further. However, our data are consistent with a report of an association between HHC and late onset of AIDS (> 10 years) in a cohort of Japanese hemophiliacs [34]. Time-dependent haplogroup effects on disease progression may also exist, as observed for the *CCR2b-64I* and

CCR5-Δ*32*-bearing haplogroups HHF⋆2 and HHG⋆2, respectively [28]. Studies of the associated alleles have demonstrated that protection in *CCR2b-64I* carriers is greatest early in the course of infection [12,14,35], whereas the benefit to carriers of *CCR5*-Δ*32* appears to diminish 2 years after the diagnosis of AIDS [14]. To investigate a possible time-dependent effect for HHC, further studies will be conducted after the accumulation of additional follow-up time.

It is interesting to note that this is the first study involving a population with two HIV-1 subtypes (B and CRF01_AE) co-circulating. We found that IDU carrying either the *CCR5-2554T* or *CCR5-2086G* allele were more likely to be infected with CRF01_AE than those infected with subtype B ($P < 0.001$). It is plausible that subtype-specific biological characteristics may account for this observed difference in allelic frequency distributions, since our earlier characterization of subtype B and CRF01_AE viruses from this cohort revealed major intersubtype differences in the proportions of different envelope V3 motifs as well as predicted coreceptor usage and phenotype from genetic sequence data [36].

The identification of polymorphisms affecting progression to AIDS and the mechanisms by which they mediate their effects in different populations will be an important step in the development of vaccines and in improving treatment strategies. Haplogroup-mediated effects, such as the effect on antiretroviral therapy among persons with the *CCR5*-Δ*32*-bearing haplogroup, have already been examined [37–41], and these investigations suggest that genotyping may help clinical decision making. The continued analysis of *CCR5* haplogroups and their effects on disease progression among different ethnic populations will provide useful information for medical advances and could one day have a profound impact on public health.

In summary, we report the first *CCR5* human haplogroup associations with HIV-1 disease progression in an Asian population. This study highlights the possibly complex interaction between haplogroups, ethnicity and HIV-1 disease progression. Clearly, additional studies in non-Caucasian populations may help to elucidate these interactions. The identification of ethnic group-specific genetic effects may also provide valuable insights into the differences observed in transmission and pathogenesis of HIV-1 globally and for identifying novel approaches to treatment and prevention.

Acknowledgements

The authors gratefully thank the participants of the study and all of the staff affiliated with this study from the BMA, Thailand MOPH-US CDC Collaboration, Mahidol University, and the Centers for Disease Control and Prevention for administrative, clinical, laboratory, and data management support. Specifically, we would like to acknowledge the study coordinators from BMA and Thailand MOPH-US CDC Collaboration. In addition, we would like to thank Dr Roman Gvetadze for statistical and data management support.

Sponsorship: This work was partially supported by Emerging Infections Funding for Genetic Working Group of CDC.

Note: Use of trade names is for identification only and does not constitute endorsement by the US Department of Health and Human Services, the Public Health Service, or the Centers for Disease Control and Prevention.

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