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

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\textbf{Background:} An evolutionary-based analysis of the CC chemokine receptor 5 gene (\textit{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.

\textbf{Objective:} To assess the influence of \textit{CCR5} haplogroups on HIV-1 disease progression in a Thai population.

\textbf{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).

\textbf{Methods:} From a cohort of 130 IDU, 106 (81.5\%) were genotyped for the \textit{CCR2} textsuperscript{b}-64I, \textit{CCR5} textsuperscript{32} and seven \textit{CCR5} promoter alleles constituting the \textit{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 \texttimes \ 10\textsuperscript{3} cells/l.

\textbf{Results:} The most common \textit{CCR5} haplogroups were HHC (61.8\%), followed by HHE (15.6\%) and HHE*2 (14.6\%). HHE was associated with an accelerated CD4 count decline to < 200 \texttimes \ 10\textsuperscript{3} cells/l (adjusted relative hazard, 1.88; 95\% confidence interval, 1.05–3.36; \( P = 0.02 \)).

\textbf{Conclusions:} This is the first evidence that the \textit{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.

\textbf{Introduction}

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

\textbf{Keywords:} Asia, HIV-1, \textit{CCR5}, \textit{CCR2}, host genetics

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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 seroconverters (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 BsaI (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 × 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 × 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 CCR5-2086G (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>
<thead>
<tr>
<th>Allele</th>
<th>Genotype</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR2b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>64V</td>
<td>VN</td>
<td>181 (85.4)</td>
</tr>
<tr>
<td>64I</td>
<td>VI</td>
<td>31 (14.6)</td>
</tr>
<tr>
<td>CCR5-Δ32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>+/-</td>
<td>212 (100.0)</td>
</tr>
<tr>
<td>Deletion</td>
<td>0/0</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>CCR5P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-2733A</td>
<td>A/A</td>
<td>209 (98.6)</td>
</tr>
<tr>
<td>-2733G</td>
<td>A/G</td>
<td>3 (1.4)</td>
</tr>
<tr>
<td>-2554G</td>
<td>G/G</td>
<td>81 (38.2)</td>
</tr>
<tr>
<td>-2554T</td>
<td>G/T</td>
<td>131 (61.8)</td>
</tr>
<tr>
<td>-2459G</td>
<td>G/G</td>
<td>144 (67.9)</td>
</tr>
<tr>
<td>-2459A</td>
<td>G/A</td>
<td>68 (32.1)</td>
</tr>
<tr>
<td>-2135T</td>
<td>T/T</td>
<td>144 (67.9)</td>
</tr>
<tr>
<td>-2135C</td>
<td>T/C</td>
<td>68 (32.1)</td>
</tr>
<tr>
<td>-2132C</td>
<td>C/C</td>
<td>212 (100.0)</td>
</tr>
<tr>
<td>-2132T</td>
<td>C/T</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>-2086A</td>
<td>A/A</td>
<td>81 (38.2)</td>
</tr>
<tr>
<td>-2086G</td>
<td>A/G</td>
<td>131 (61.8)</td>
</tr>
<tr>
<td>-1835C</td>
<td>C/C</td>
<td>180 (84.9)</td>
</tr>
<tr>
<td>-1835T</td>
<td>C/T</td>
<td>32 (15.1)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/C</td>
<td>7 (6.6)</td>
</tr>
<tr>
<td>A/E</td>
<td>3 (2.8)</td>
</tr>
<tr>
<td>A/F*1</td>
<td>1 (0.9)</td>
</tr>
<tr>
<td>A/F*2</td>
<td>1 (0.9)</td>
</tr>
<tr>
<td>A/G*1</td>
<td>1 (0.9)</td>
</tr>
<tr>
<td>C/C</td>
<td>38 (35.9)</td>
</tr>
<tr>
<td>C/E</td>
<td>19 (17.9)</td>
</tr>
<tr>
<td>C/F*2</td>
<td>28 (26.4)</td>
</tr>
<tr>
<td>C/G*1</td>
<td>1 (0.9)</td>
</tr>
<tr>
<td>E/E</td>
<td>5 (4.7)</td>
</tr>
<tr>
<td>E/G*1</td>
<td>1 (0.9)</td>
</tr>
<tr>
<td>F<em>2/F</em>2</td>
<td>1 (0.9)</td>
</tr>
</tbody>
</table>

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.

There was a significant association between HHE and rapid CD4 T lymphocyte count decline to < 200 × 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...
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).

**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
Among persons with the effects, such as the effect on antiretroviral therapy, improving treatment strategies. Haplogroup-mediated transmission to AIDS and the mechanisms by which they affect antiretroviral therapy, improving treatment strategies. Haplogroup-mediated transmission to AIDS and the mechanisms by which they 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-Δ32-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.

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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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