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Association between HIV-1 tropism and *CCR5* human haplotype E in a Caucasian population

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Abstract

Background—The influence of the diversity of *CCR5* on HIV susceptibility and disease progression has been clearly demonstrated but how the variability of this gene influences the HIV tropism is poorly understood. We investigated whether *CCR5* haplotypes are associated with HIV tropism in a Caucasian population.

Methods—We evaluated 161 HIV-positive subjects in a cross-sectional study. *CCR5* haplotypes were derived after genotyping nine *CCR2-CCR5* polymorphisms. The HIV subtype was determined by phylogenetic analysis using the Maximum Likelihood method and viral tropism by the genotypic tropism assay (geno2pheno). Associations between *CCR5* haplotypes and viral tropism were determined using logistic regression analyses. Samples from 500 blood donors were used to evaluate the representativeness of HIV-positives in terms of *CCR5* haplotype distribution.

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Results—The distribution of *CCR5* haplotypes was similar in HIV-positive subjects and blood donors. The majority of viruses (93.8%) belonged to HIV-1 CRF06_cpx; 7.5% were X4, and the remaining were R5 tropic. X4 tropic viruses were overrepresented among people with *CCR5* human haplotype E (HHE) compared to those without this haplotype (13.0% vs 1.4%; $p=0.006$). People possessing *CCR5* HHE had eleven times increased odds ($OR=11.00$; 95% CI 1.38 to 87.38) of having X4 tropic viruses than those with non-HHE. After adjusting for antiretroviral therapy (ARV), neither the presence of HHE nor the use of ARV were associated with X4 tropic viruses.

Conclusions—Our results suggest that *CCR5* HHE as well as ARV treatment might be associated with the presence of HIV-1 X4 tropic viruses.

Keywords

CCR5; X4 tropic viruses; intravenous drug users

Introduction

Human immunodeficiency virus type 1 (HIV-1) uses chemokine receptors (cysteinecysteine receptor 5 [*CCR5*] and/or cysteine-X-cysteine receptor 4 [*CXCR4*]) as coreceptors to enter CD4 expressing cells ¹. Which of these co-receptors is used is mainly determined by the amino acid sequences of the V3 loop of gp120. Accordingly, based on co-receptor usage, HIV-1 may be classified into R5 tropic (using *CCR5*), X4 tropic (using *CXCR4*), or dual/mixed tropic (using both receptors) ². Categorization of X4 tropic viruses as syncytium-inducing (SI) and R5 as non-syncytium-inducing (NSI) is often used but in fact these features, although highly correlated, are not identical ².

In the early stages of HIV infection R5 tropic viruses predominate, whereas X4 tropic viruses emerge with the progression of the disease ³. Consequently, studies suggest that X4 tropic viruses are associated with an increase in viral load, a decrease in CD4+ T cell counts, and rapid progression to AIDS and death ^{1, 3, 4}.

Genetic diversity in the *CCR5* receptor encoding gene is associated with susceptibility to HIV infection and disease progression ⁵. The best described mutation, a 32 base-pair deletion (*CCR5*-32) in the open reading frame of *CCR5* in the homozygous state provides complete resistance to the acquisition of R5 tropic HIV by truncating receptor expression on the cell surface ⁶. In addition, there are other single nucleotide polymorphisms (SNPs) in the *CCR2* and *CCR5* cis-regulatory region based on which the human haplotypes (HH) A-E, F*1, F*2, G*1 and G*2 have been defined using evolutionary-based analysis (**Figure 1**) ^{7, 8}.

Some of the *CCR5* haplotypes (e.g. HHE) are likely associated with the acceleration of disease progression but the exact mechanism is still largely unknown ^{7, 9}. Possible associations between *CCR5* diversity and co-receptor usage have been poorly studied. Recently, Crudeli *et al* demonstrated that the presence of SI viruses was associated with *CCR5*-32 genotype (i.e. HHG*2) at the acute phase but not in the later stages of HIV infection ¹⁰. Another study has shown that *CCR5* HHE/HHF*2 was associated with the presence of SI viruses ¹¹.

Taking into consideration the importance of *CCR5* diversity in the acquisition and progression of HIV infection on the one hand, and the relevance of HIV-1 tropism to disease progression and prognosis on the other, we aimed to investigate whether and how *CCR5* haplotypes relate to the tropism of HIV-1 in a Caucasian population predominantly infected via intravenous drug use.

Material and methods

In a cross-sectional study, Caucasian HIV-positive volunteers (N = 161) were recruited from Estonian syringe-exchange programs and from three Estonian prisons in 2006 and 2007. Participants were screened for HIV using a fourth generation enzyme-linked immunoassay (Abbott IMx HIV-1/HIV-2 III Plus, Abbott Laboratories, Abbott Park, Illinois, USA) and the results were confirmed by immunoblot assay (INNO LIA HIV I/II Score Westernblot (Microgen Bioproducts Ltd, Surrey, UK) in the Estonian Central HIV Reference Laboratory. In addition, blood samples (from residual blood left after routine testing) from 500 HIV-, HBV- and HCV-negative Estonian blood donors were analyzed to confirm the representativeness of HIV-positives in terms of distribution of *CCR5* haplotypes.

Genomic DNA was extracted from whole blood or peripheral blood mononuclear cells using a Qiagen QIAamp DNA minikit (Qiagen, Hilden, Germany). *CCR5* polymorphisms A29G, G208T, G303A, T627C, C630T, A676G, C927T and the presence of *CCR5* 32 and *CCR2*-V64I (G190A) were detected by the Taqman Allelic Discrimination assay (Applied Biosystem, Carlsbad, CA, USA). An evolutionary-based classification of *CCR5* polymorphisms was used to define the *CCR5* haplotypes as described elsewhere (Figure 1)^{7, 8}.

HIV RNA was extracted from 140 µl plasma using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. HIV-1 V3 region was amplified using seminested reverse transcription (RT)-PCR as described elsewhere¹². In brief, viral RNA was reverse transcribed into cDNA using 1x RT reaction buffer, 1 µM dNTP, 16U M-MuLV RT, 16U RNase inhibitor, and 0.4 µM antisense primer JA170 (5'-GTGATGTATTTCARTAGAAAAATTC-3'). V3 region was amplified in a first round PCR using 1x HotStart Buffer, 1.5 mM MgCl₂, 0.2 µM dNTP, one unit of 6:1 mixture of Taq and Pfu DNA polymerase, 0.3 µM primer JA168 (5'-ACAATGYACACATGGAATTARGCCA-3') and 0.3 µM primer JA170. The PCR program consisted of 40 cycles of 95 °C for 30 sec, 40 °C for 30 sec and 72 °C for 30 sec. The second round PCR was carried out in the same conditions as the first round using primers JA168 and JA169 (5'-AGAAAAATTCYCCTCYACAATTAAA-3') and 30 cycles. All RT and PCR reagents were purchased from Fermentas (Vilnius, Lithuania). All second round PCR products were directly sequenced using the ABI Prism Big Dye 3.0 fluorescent terminator sequencing chemistry (Applied Biosystems, Foster City, CA) with the second round PCR primers.

V3 sequences were assembled to contigs with the Vector NTI software (Life Technologies, Paisley, UK). Alignments were conducted by MEGA 5 (www.megasoftware.net) software packages and a phylogenetic tree was constructed using the Maximum Likelihood method

based on the General Time Reversible model^{13,14}. All reference sequences were taken from the Los-Alamos HIV Sequence Database (www.hiv.lanl.gov). HIV-1 tropism was determined by geno2pheno analysis (www.geno2pheno.org) by liberal criteria using a false positive rate (FPR) of 20% as suggested previously¹⁵.

The program R 2.13.1 (www.r-project.org last accessed 1st January 2012) was used for statistical analyses. The distribution of X4 tropic virus between different *CCR5* haplotypes, and between the subgroup receiving antiretroviral (ARV) treatment and the untreated subgroup were compared with Fisher's exact test. Univariate and multivariate logistic regression models were constructed to determine the associations between *CCR5* haplotypes, viral tropism, and ARV treatment.

The Ethics Committees of Tallinn and Tartu approved this study. All HIV-positive subjects gave informed consent to take part in the study. Blood donors agreed to the use of residual blood for research purposes.

Results

The HIV-positive population (n = 161) was characterised by the predominance of young, male, intravenous drug users (IDUs) (80%), and by the high rate of co-infection with HCV (86%) (**Table 1**).

The HIV-1 subtypes were determined by phylogenetic analyses which revealed the monophyletic pattern of the V3 region of HIV-1 which is consistent with our previous studies^{16, 17}. Of the sequenced viruses, 93.8% belonged to CRF06_cpx. Two viruses were found to be of subtype B and four were A1, while four viruses remained unclassified (data not shown). Using the geno2pheno algorithm, 12/161 viruses (7.5%) were determined to be X4 tropic and the remaining viruses were R5 tropic.

While analysing viral tropism in terms of ARV treatment, we observed that X4 tropic viruses were overrepresented in ARV-treated compared to treatment-naive subjects [23% (3/13) vs 4.7% (6/127), respectively; p=0.038]. The ARV treatment history of three subjects with X4 tropic viruses was unknown. A univariate logistic regression model revealed associations between ARV treatment and the presence of X4 tropic viruses (odds ratio [OR] = 6.05; 95% Confidence Interval [CI] 1.31 to 27.90; p=0.021) but no other factors (gender, age, transmission route, or duration of intravenous drug use) were associated with the occurrence of X4 tropic viruses (data not shown).

In total, 160 HIV-positive subjects and 500 blood donors were successfully genotyped for *CCR2-CCR5* polymorphisms and classified into haplotypes. The most common *CCR5* haplotypes were HHE and HHC, followed by HHA in HIV-positives and by HHF*2 in blood donors. No subjects were categorised as HHB or HHD (**Figure 1**). In total, 20 different *CCR5* haplotype pairs were described. The most common pairs were combinations of HHC and HHE in HIV-positive subjects (HHC/HHE – 18.8%, HHE/HHE – 11.9% and HHC/HHC – 11.3%) and HHC/HHE (18.4%), HHE/HHE (10.8%) and HHE/HHF*2 (10.0%) in blood donors. There were no differences in the distribution of *CCR5* haplotypes

or haplotype pairs between HIV-positive subjects and blood donors showing the representativeness of the HIV-positive population.

We next evaluated the distribution of X4 tropic viruses between people with different *CCR5* haplotypes (**Figure 2**) and observed an overrepresentation of X4 tropic viruses only in those with *CCR5* HHE compared with those with *CCR5* non-HHE haplotypes (13.0% vs 1.4%, respectively; $p=0.006$). In a univariate logistic regression model people possessing *CCR5* HHE had eleven times increased odds of having X4 tropic viruses compared with those with non-HHE (OR=11.00; 95% CI 1.38 to 87.38). In the multivariate model neither *CCR5* HHE nor ARV treatment was associated with the presence of X4 tropic viruses (**Table 2**). There were no differences in the distribution of X4 tropic viruses between *CCR5* haplotype pairs.

Discussion

In a study predominantly involving Caucasian IDUs and looking at the associations between HIV-1 tropism and *CCR5* human haplotypes we provide evidence that, together with ARV treatment, the possession of *CCR5* HHE might be associated with the out-selection of X4 tropic viruses. However, neither of these factors remained independently associated in a multivariate model suggesting either an equal or combined effect of these two factors. While the associations between ARV treatment and the presence of X4 viruses (probably due to more advanced phase of infection) have been described previously³ the role of *CCR5* haplotypes in this process is much less studied.

Interestingly, both *CCR5* HHE and HIV-1 X4 tropic viruses have been related to a poor outcome of HIV infection in subjects receiving ARV treatment^{3, 7, 9}. The *CCR5* HHE has also been associated with increased susceptibility to HIV and with more rapid disease progression in European, American, Argentinian, and Japanese populations^{7, 9, 18, 19}. Despite of that only a limited number of studies have explored the relationships between the diversity of the *CCR5* encoding gene and the prevalence of X4 tropic viruses. Crudeli *et al* demonstrated that people who were *CCR5*- $\delta 32$ heterozygotes (HHG*2) had higher rates of X4 tropic viruses than those not having this genotype in the acute phase of infection¹⁰. Altamirano *et al* demonstrated that all wild type homozygotes for *CCR5* SNP G303A had NSI virus while all SI variants were found in individuals with A/A or A/G genotypes (HHE-HHF*2)¹¹. Similarly, we have shown in this study that almost all X4 tropic viruses (11/12) occurred in individuals with *CCR5* HHE, the haplotype that, among others, bears an A/A or A/G genotype at position 303 (**Figure 1**).

The mechanism of how X4 tropic viruses are related to disease progression in HIV infection is poorly understood^{20, 21}. It is still unclear whether the increased frequency of X4 tropic viruses is the cause or the consequence of disease progression. Studies have shown a switch from R5 to X4 tropic viruses in the presence of *CCR5* antagonists. After the discontinuation of these agents the process is reversed – X4 tropic viruses rapidly disappeared and resided only as a minority population^{22, 23}. This raises the question of which viral and/or host factors control the switch from R5 to X4 tropic viruses. The present study indicates that host factors such as *CCR5* haplotypes might partly explain relationships between viral tropism

and HIV disease progression. We suggest that individuals with *CCR5* HHE may be more likely to have X4 tropic viruses than those with other haplotypes.

As the *CCR5* density correlates with viral load, disease progression, and response to treatment, it is hypothesised that X4 tropic viruses emerge as a consequence of a reduction of *CCR5* expression or due to the over-expression of CXCR4 on the cell surface²⁴⁻²⁶. The relationship between the expression of *CCR5* and CXCR4 is demonstrated by the finding that *CCR5*-32 heterozygotes have lower *CCR5* density and smaller numbers of cells expressing *CCR5* but at the same time have an increased density of CXCR4 on CD3+ cells compared to *CCR5* wild type homozygotes²⁷. They also showed that *CCR5*-32 heterozygotes had lower plasma concentrations of the CXCR4 ligand, SDF-1 α , suggesting an interaction between these two chemokine receptors and a possible influence on HIV tropism. Therefore, it could be presumed that changes in the expression of these receptors may have an impact on these interactions.

One could further assume that the presence of *CCR5* HHE might contribute to increased *CCR5* expression and, through that, to the out-selection of X4 tropic viruses. On the other hand, however, the evidence supporting this assumption is not straightforward as most studies have explored *CCR5* SNPs and not the haplotypes. It has been demonstrated that haplotypes HHA-HHE are associated with higher *CCR5* transcriptional activity^{8, 28}. Individuals who are homozygous for 303A (HHE-HHG*2) have higher *CCR5* density on CD14+ monocytes and a higher number of CD4+ T cells expressing *CCR5* compared to other genotypes^{29, 30}. More recently, Picton and co-authors (2012) in a cohort of South Africans and Caucasians failed to demonstrate any association between the presence of HHE and *CCR5* expression³¹. Thus, drawing conclusions on haplotypes based on SNP studies alone is difficult if not impossible.

One characteristic of our study was the high prevalence of IDUs among participants. Previous studies have shown that epithelial cells from the rectum, endocervix, and vagina produce high levels of SDF-1 α , a ligand of CXCR4, which inhibits X4 tropic viruses and thus gives a preference to R5 tropic HIV acquired via sexual, but not intravenous, transmission³². One might therefore predict that the rate of infection with X4 tropic viruses would be greater among IDUs than among those who acquired HIV sexually. The limited studies conducted thus far have given conflicting results. Spijkerman et al (1995) demonstrated a significantly lower proportion of infections with X4 viruses among IDUs compared to men who had sex with men³³. Other studies have shown the opposite result³⁴⁻³⁶. We observed that all X4 tropic viruses were among IDUs but it was not significant. However, we noticed that IDUs clearly dominated over sexually infected subjects and we observed a lower rate of infection with X4 viruses (7.5%) than in other studies (36% to 53%)³⁴⁻³⁶.

Some limitations of the study should be noted. First, the stage of HIV disease, the time from HIV diagnosis, the type and the length of ARV treatment, and the viral load and CD4+ counts were not collected. This prevented us from correlating the prevalence of X4 viruses with the indices of disease progression. Second, details of ARV treatment history were not reported in 13% of the study population, which may have decreased the number of X4 tropic

viruses in analyses where all co-variables (tropism, ARV history and *CCR5* haplotypes) were included. Another critical point was the choice of tropism assay. As in other studies, phenotypic assays could not be used because of their high costs and complex logistics. Therefore we selected a geno2pheno assay as recommended by the European guidelines for clinical HIV-1 tropism testing³⁷. However, we acknowledge that most genotypic assays are developed in subtype B viruses rather than CRFs; the latter constituted more than 90% of the viruses in our study. Despite this, we believe that these limitations do not prevent us from drawing reasonably firm conclusions

In summary, we have demonstrated that the possession of *CCR5* HHE as well as ARV treatment could be related to the presence of X4 tropic viruses in Caucasian HIV-positive subjects. However, we wish to stress that neither of these factors was independently related and the pathogenetic mechanism remains to be elucidated in further studies. Nevertheless, we have demonstrated the influence of host genetics on the virus-host interaction during HIV infection, and suggest that differences in viral tropism could be at least partly explained by human genetic factors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

CCR5 haplotypes by evolution based on *CCR2* and *CCR5* polymorphisms and the distribution of *CCR5* haplotypes among HIV-positive subjects and blood donors. NOTE. On the basis of the linkage disequilibrium patterns between the polymorphisms in the coding (–32) and noncoding (promoter) region of *CCR5* and the coding polymorphism (V64I) in *CCR2*, an evolutionary-based strategy has been used to generate the *CCR5* human haplotypes (HH) shown below the *CCR5* gene structure⁸. These *CCR5* HH are designated as HHA to HHG*2, with HHF*2 and HHG*2 denoting the haplotypes that bear the *CCR2*-64I and *CCR5*-32 polymorphisms, respectively. Because of its similarity to the chimpanzee *CCR5* sequence, the human *CCR5* HHA haplotype is classified as the ancestral *CCR5* haplotype⁸. Nucleotide variations relative to the ancestral sequence are shown. The *CCR5* numbering systems used in the literature are shown. Top numbering is based on GenBank accession numbers AF031236 and AF031237; middle numbering is based on GenBank accession number U95626; bottom numbering is the numbering system in which the first nucleotide of the *CCR5* translational start site is designated as +1 and the nucleotide immediately upstream as –1⁸. ORF, *CCR5* open-reading frame; Wt, wild-type; D32, 32 base-pair deletion.

Figure 2.

The distribution of X4 and R5 tropic viruses in individuals with different *CCR5* haplotypes compared to those who do not possess the particular haplotype.