X4 Tropic Virus Prediction Is Associated with a Nadir CD4 T-Cell Count below 100 Cells/mm³

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Key Words

Tropism · Human immunodeficiency virus · Algorithm · CD4 nadir · CCR5 · Maraviroc

Abstract

Objective: The aim of this study was to evaluate tropism prediction performances of three algorithms [geno2pheno false-positive rate 10% (G2P10), position-specific scoring matrix (PSSM) and a combination of the 11/25 and net charge rules] and to investigate the viral and host factors potentially involved in the X4 or R5 prediction in human immunodeficiency virus-1 (HIV-1) patients. **Methods:** Viral tropism was determined in 179 HIV-1-infected patients eligible for CCR5 antagonist therapy. HIV-1 RNA or DNA was extracted and amplified for *env* gp120 sequencing. In parallel, demographic, viral, immunological and clinical determinants were analyzed. **Results:** According to the G2P10 algorithm, 48 patients harbored X4 or X4R5 virus. The tropism prediction was concordant for 87.7 and 88.2% of samples when comparing G2P10 with PSSM or with a combination of the 11/25 and net

charge rules, respectively. X4 prediction was significantly associated with more than 35 amino acids in the V3 domain ($p < 0.0001$) and loss of an N-linked glycosylation site ($p < 0.0001$). Of the factors studied, only the nadir CD4 T-cell count was significantly associated with X4 tropism ($p = 0.01$). **Conclusion:** We determined that the X4 virus detection is closely linked to the nadir CD4 T-cell count below 100 cells/mm³ that must be taken into account when considering a CCR5 antagonist therapy switch.

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Introduction

Since the introduction of the specific CCR5 antagonist maraviroc into clinical practice, laboratory-based and clinical studies have focused on coreceptor usage by human immunodeficiency virus type 1 (HIV-1). Determining HIV coreceptor tropism is a prerequisite for the initiation of maraviroc treatment because HIV-1 can use the CCR5 and the CXCR4 membrane receptors (R5 and X4

viruses, respectively) or both (dual/mixed virus) to enter target cells. Although HIV-1 coreceptor tropism can be measured phenotypically with a few, highly complex, standardized assays, most patient screening for maraviroc treatment in Europe is based on genetic tropism testing (the amplification and sequencing of the V3 domain of the viral envelope) [1]. Initially, the '11/25 rule' and the 'net charge rule' were used to predict tropism from the V3 sequence but showed only a moderate correlation with the results of phenotypic assays [2, 3]. Although the results of specific genotyping tools [such as geno2pheno and position-specific scoring matrix (PSSM)] are similar to those of the phenotypic assays, selection of a specific cutoff for the false-positive rate for geno2pheno is determinant in the chance of falsely predicting a virus as an X4 virus.

The selective pressure that leads to the emergence of either X4 or R5 virus is complex, and its relevance in the pathogenesis and natural history of HIV is still only partially understood [4–6]. Some studies suggest that the X4 virus is more pathogenic than the R5 virus because X4 is associated with faster decline in the CD4+ cell count, a high risk of clinical disease [7] and a lower baseline CD4+ cell count [8]. Recently, Soulié et al. [9] have shown that virus tropism has no impact on the change over time in CD4+ cell count in HIV-1 patients with >350 CD4+ cells/mm³ at diagnosis. Taken as a whole, these findings suggest that other factors related to disease progression merit investigation.

Hence, we decided to analyze HIV-1 tropism in 179 HIV-1-infected patients eligible for anti-CCR5 therapy by using 3 different algorithms [geno2pheno false-positive rate 10% (G2P10), PSSM and a combination of the 11/25 and net charge rules] and investigate the viral and host factors potentially implicated in the X4 or R5 prediction.

Materials and Methods

Study Population

Samples of plasma or peripheral blood mononuclear cells were obtained from 179 HIV-1-infected patients eligible for anti-CCR5 therapy. Following the provision of written, informed consent, patients were enrolled by 6 tertiary medical centers in the Picardy region of northern France from October 2010 to February 2013. Demographic and clinical data (age, gender, HIV risk factors, duration of HIV infection, antiretroviral exposure, CD4 nadir, current CD4 count, CD4/CD8 ratio and HIV RNA load) were collected and analyzed.

Prediction of Viral Tropism

HIV-1 RNA or DNA was extracted and amplified from plasma or peripheral blood mononuclear cells for *env* gp120 sequencing.

Briefly, V3 *env* was amplified and bulk sequenced. HIV-1 coreceptor usage was determined from the V3 *env* sequence using the G2P10 and PSSM bioinformatics tools with the syncytium-induc-

ing/non-syncytium-inducing and X4/R5 matrices. Geno2pheno and PSSM are available at <http://coreceptor.bioinf.mpi-inf.mpg.de/> and <http://indra.mullins.microbiol.washington.edu/webpssm/>, respectively.

We also combined the genotypic rules based on the amino acid (aa) residues at V3 positions 11 and 25 (the 11/25 rule) and the overall net charge of the V3 sequence (the net charge rule) to predict HIV-1 tropism. One of the following criteria had to be met when predicting CXCR4 coreceptor usage: (i) R or K at position 11 of V3 and/or K at position 25, (ii) R at position 25 of V3 and a net charge of $\geq +5$, or (iii) a net charge of $\geq +6$. Viruses were classified as R5 (R5) or X4-tropic (X4/R5X4).

Statistical Analysis

The characteristics of patients classified as harboring an R5 or non-R5 virus were compared and tested for statistical significance using the nonparametric Mann-Whitney test for continuous variables and χ^2 or Fisher's exact test for categorical variables. A 2-sided *p* value of ≤ 0.05 was considered statistically significant.

Results

A total of 179 samples (subtype B: 114; 63.7%) from HIV-1-infected patients (110 men and 69 women; mean age $\pm$ standard deviation: 43.6 ± 10.3 years) were analyzed. The mean CD4 T-cell count was 448 ± 298 cells/mm³, and 17.8% of the patients had a count below 200 cells/mm³. The mean plasma HIV-1 RNA was 3.17 ± 1.67 log₁₀ HIV-1 RNA copies/ml. 17.8% of the individuals were antiretroviral treatment naïve.

The 179 V3 loop sequences were analyzed for tropism using the G2P10, PSSM and a combination of the 11/25 and net charge rules. All 179 sequences were recognized by the three algorithms. Of these, 131 (73%) and 48 (27%) were classified as R5 and X4 viruses, respectively, using G2P10 (table 1). The tropism predictions were concordant for 157 (87.7%) samples when comparing G2P10 with PSSM and 158 (88.2%) when comparing G2P10 with a combination of the 11/25 and net charge rules.

The aa positions of the V3 loop sequences were also analyzed as a function of G2P10 tropism. X4 viruses were significantly associated with a V3 length >35 aa, a net charge $> +5$ and loss of V3's N-linked glycosylation site ($p < 0.0001$; table 1).

The baseline clinical, immunological and virological characteristics of the 179 patients are shown in table 2. Of the various factors studied (time since diagnosis of HIV infection, age, gender, viral load, risk factors, CDC stage, CD4/CD8 ratio, and nadir and current CD4 cell counts), only the nadir CD4 T-cell count was significantly associated with virus tropism ($p = 0.028$). The mean nadir CD4 cell count was 151 ± 125.7 cells/mm³ in patients with X4/

Table 1. Tropism prediction performances of the three algorithms and specific characteristics of V3 loop sequences as a function of the viral tropism

	G2P10		p value
	R5 (n = 131)	X4/R5X4 (n = 48)	
PSSM	145	34	
Combination of the 11/25 and net charge rules	154	25	
N-linked glycosylation site mutation	0	6 (13)	<0.0001
GAG motif mutation	17 (13)	10 (21)	0.238
Net charge >+5	2 (1.5)	15 (31)	<0.0001
Length of V3 >35 aa	0	6 (13)	<0.0001

Figures in parentheses indicate percentages.

Table 2. Baseline characteristics of the R5 and X4/R5X4 subgroups as a function of the G2P10 viral tropism

Characteristic	R5 (n = 131)	X4/R5X4 (n = 48)	p value
Mean age $\pm$ SD at sample collection, years	43 $\pm$ 11	44.2 $\pm$ 8.8	0.47
Gender, n			
Female	53 (40.4)	16 (33.3)	0.49
Male	78 (59.5)	32 (66.7)	
Mean HIV RNA, log10 copies/ml	2.91 $\pm$ 2.15	2.78 $\pm$ 1.91	0.50
Median current CD4 cell count, n/mm ³	454 $\pm$ 297	447.3 $\pm$ 306.7	0.93
Median current CD4/CD8 ratio	0.65 $\pm$ 0.56	0.51 $\pm$ 0.40	0.28
Time since diagnosis of HIV infection, n			
<10 years	61 (46.5)	21 (43.7)	0.29
>10 years	49 (37.5)	25 (52.1)	
n.d.	21 (16.0)	2 (4.2)	
Median nadir CD4 cell count, n/mm ³	235 $\pm$ 215.2	151 $\pm$ 125.7	0.02
Nadir CD4 cell count, n			
<100/mm ³	30 (22.9)	21 (43.8)	0.01
>100/mm ³	83 (63.4)	21 (43.8)	
n.d.	18 (13.7)	6 (12.4)	
Time between nadir CD4 and the determination of HIV tropism, n			
<5 years	61 (46.5)	21 (43.7)	0.35
>5 years	39 (29.8)	20 (41.7)	
n.d.	31 (23.7)	7 (14.6)	
CDC stage, n			
A	83 (63.3)	27 (56.2)	0.36
B	12 (9.2)	8 (16.7)	
C	27 (20.6)	11 (22.9)	
n.d.	9 (6.9)	2 (4.2)	
Risk factor, n			
Sexual factors	102 (77.8)	38 (79)	0.73
Parenteral factors	9 (6.9)	2 (4.2)	
n.d.	20 (15.3)	8 (16.8)	
ARV exposure (naïve), n	25 (19.1)	7 (14.6)	0.65

Figures in parentheses indicate percentages. n.d. = Not determined; ARV = antiretroviral.

R5X4 viruses and 235 ± 215.2 cells/mm³ in patients with R5 viruses. In contrast, there was no significant inter-group difference in the mean current CD4 cell count (454 ± 297 cells/mm³ for R5 and 447.3 ± 306.7 cells/mm³ for X4/R5X4 viruses; $p = 0.93$). Interestingly, a nadir CD4 cell count below 100 cells/mm³ was strongly associated with the results of tropism prediction ($p = 0.012$; table 2).

Discussion

Given that the prediction of HIV coreceptor tropism in general and R5 status in particular is a prerequisite for maraviroc treatment, we compared the predictive ability of three genotype-based algorithms that analyze the V3 loop gp120 sequence.

The overall degrees of concordance between pairs of algorithms (87.7% for G2P10 vs. PSSM and 88.2% for G2P10 vs. a combination of the 11/25 and net charge rules) were similar to previously published values [10]. However, we observed that PSSM and the combination of the 11/25 and net charge rules detected X4 viruses less frequently than G2P10 did.

We also performed a detailed analysis of each aa position in the 179 sequences as a function of the G2P10 tropism prediction. Specific V3 loop motifs (such as asparagine mutations leading to the loss of the N-linked glycosylation site, and a length >35 aa) are significantly associated with X4 prediction. A previous study has emphasized that a change in the number of aa may complicate sequence alignment and genotropism prediction with the PSSM algorithm [10]. In the present study, all sequences were correctly processed by all three algorithms. However, the association between the V3 sequence comparison and differences in tropism prediction highlighted the importance of a length >35 aa.

The presence or absence of an N-linked glycosylation site within the V3 loop reportedly influences coreceptor

tropism [11, 12]. It has been demonstrated that glycans are covalently linked to specific asparagine residues within the envelope precursor protein gp160 by the host cell machinery and are important for the correct, stable folding of gp120. The results of a recent study suggested that the presence of N-linked glycans has a significant impact on the dynamics of the gp120 protein (particularly the movement and preferred conformation of the V3 loop) and that N-linked glycosylation has a role in determining HIV-1 coreceptor tropism [13]. In the present study, mutations of the asparagine residues in the V3 loop were significantly correlated with X4 prediction ($p < 0.0001$).

Lastly, we analyzed demographic, virological, immunological and clinical determinants. Of the various factors studied, only the nadir CD4 T-cell count alone was significantly associated with X4 tropism ($p = 0.01$). The association was especially strong for a nadir count below 100 cells/mm³ ($p = 0.02$). In contrast, current CD4 cell counts were not significantly associated with X4 tropism. A longitudinal study of the emergence of X4 virus in seroconverters for more than 10 years has shown that there is a relationship between the emergence of X4 or X4/R5 virus and disease progression (as evidenced by a decrease in CD4 T-cell count) [14]. However, it has also been reported that the presence of X4 virus in HIV-1-infected patients with >350 CD4+ cells/mm³ at diagnosis has no impact on the change over time in the CD4+ cell count [9]. Although the mean CD4 T-cell count was 448 ± 298 cells/mm³ in our study population, the presence of X4 virus was closely associated with the nadir CD4 T-cell count value. The latter might be of value as an additional marker in patient care.

In conclusion, it has not yet been definitively established whether X4 viruses have a role in the pathogenesis of HIV but the nadir CD4 cell count appears to be a key marker and must be taken into account when considering the initiation of CCR5 antagonist therapy.

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