Characteristics of HIV-1 coreceptor tropisms in blood and cellular subsets of HIV-1-infected Koreans

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Department of Medicine

The Graduate School, Yonsei University

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Directed by Professor Jun Yong Choi

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Je Eun Song

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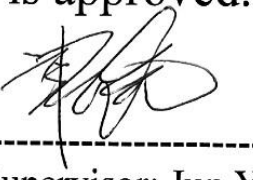
This certifies that the Doctoral Dissertation
of Je Eun Song is approved.



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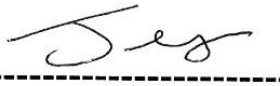

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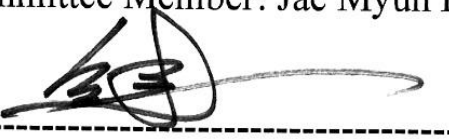

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“What does not kill me makes me stronger”

Friedrich Nietzsche

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ABSTRACT

Characteristics of HIV-1 coreceptor tropisms in blood and cellular subsets of HIV-1-infected Koreans

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Human immunodeficiency virus (HIV) is a sexually transmitted disease which causes progressive failure of the immune system. When a virus enters a cell, coreceptors CCR5 or CXCR4 are required for infection to occur. The discovery of these two coreceptors has improved scientific understanding of the interaction between the HIV and host cells, and has facilitated the development of new therapeutic approaches. Maraviroc, a CCR5 antagonist, has been developed and seen as a potential HIV-1 treatment. However, despite treatment with this compound, viral eradication remains an obstacle because; the virus remains in tissue and cellular subsets (CSs), known as ‘reservoirs’. In addition, because HIV-1 is highly mutable, it shows various features in an individual. This study aims to estimate the prevalence of the CCR5 tropism among HIV-1 infected Koreans and to compare clinical characteristics between CCR5 and non-CCR5 groups. Through single genome sequencing of HIV-1 from each CS, different coreceptor usage in cells thought to be viral resevoirs was analyzed before and after combination antiretroviral therapy (cART).

In part 1 of this study, HIV-1 infected Koreans were enrolled from four medical centers in three Korean cities between April 2013 and May 2014. Nested polymerase chain reaction and population-based sequencing for the V3 region (HXB2 position

6225-7758) of the envelope were performed with HIV RNA or proviral DNA. Genotypic tropism was determined with a web-based bioinformatics tool, geno2pheno (http://coreceptor.geno2pheno.org). In part 2 of this study, between January and December 2012, blood collected from treatment-naïve HIV-1 patients directly prior to cART initiation and again six months into treatment. Naïve $CD4^+$ T cells, resting central memory and effector memory $CD4^+$ T cells, activated $CD4^+$ T cells, monocytes, and natural killer cells were sorted using a fluorescence-activated cell sorter. HIV-1 *env* C2V3 sequences from HIV RNA in plasma and HIV DNA in CSs were generated using single genome sequencing. Genotypic tropism testing was performed using geno2pheno.

In part 1 of this study, data from 143 subjects were analyzed. The prevalence of CCR5 tropism was 69.2% (N=99). There were no significant clinical or epidemiological predictors for CCR5 tropism. In part 2 of this study, the clinical characteristics and corecptor use of five treatment-naïve patients were analyzed. Coreceptor tropism of plasma and CSs were analyzed. In three patients, tropism discordance was found in plasma and CSs.

The prevalence of CCR5 tropism in Korean HIV-1 infected individuals was not significantly different from that of previous studies. There were no different clinical factors between CCR5 and non-CCR5 groups. Thus, coreceptor tropism could not be predicted using clinical data. Tropism discordance was observed before and after treatment in various CS which have important roles as reservoirs

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Key words: human immunodeficiency virus, coreceptor tropism, cellular subset

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I. INTRODUCTION

Human immunodeficiency virus (HIV) is a member of the lentivirus subfamily of Retroviridae viruses and it is the only lentivirus known to infect humans. HIV is a sexually transmitted virus that causes progressive failure of the immune system. There are two types of HIV: HIV-1 and HIV-2. HIV-1 was the first type discovered and can be found in both lymphadenopathy associated virus and human T-lymphotropic virus 3. HIV-1 is more virulent and more infective than HIV-2, and it is the main type of HIV infection globally[1].

The HIV virion enters the $CD4^+$ T cells by adsorption of glycoproteins on its

surface to receptors on the target cell. The entry of HIV-1 into cells occurs by the interaction of the gp120 glycoprotein of the HIV-1 envelope (*env*) with cellular CD4 and a chemokine coreceptor[2]. The HIV-1 fusion reaction begins as the CD4 binding domain of gp120 adheres to the CD4 receptor and then binds to and fuses with two major coreceptors, CCR5 and CXCR4, to enter the target cells. Based on their coreceptor use, HIV strains are classified as CCR5-tropic (R5), CXCR4-tropic (X4), or dual-tropic (R5 / X4) strains[3]. The discovery of CCR5 and CXCR4 which are the two main coreceptors for HIV has facilitated the development of new therapeutic approaches. Maraviroc, the first licensed CCR5 receptor antagonist, is a promising alternative option for patients who are resistant to current combination antiretroviral therapy (cART). It targets a host coreceptor critical for HIV entry into $CD4^+$ T cells and monocyte/macrophages[4] (Figure 1). Because the compound is only effective in the R5 strain, it is important to characterize the coreceptor tropism before using maraviroc. In Korea, CCR5 antagonists have not yet been commercialized. It is essential to understand the status of CCR5-tropic HIV patients in order to select an appropriate treatment. It is also necessary to understand the epidemiology and clinical characteristics of coreceptor usage before using maraviroc.

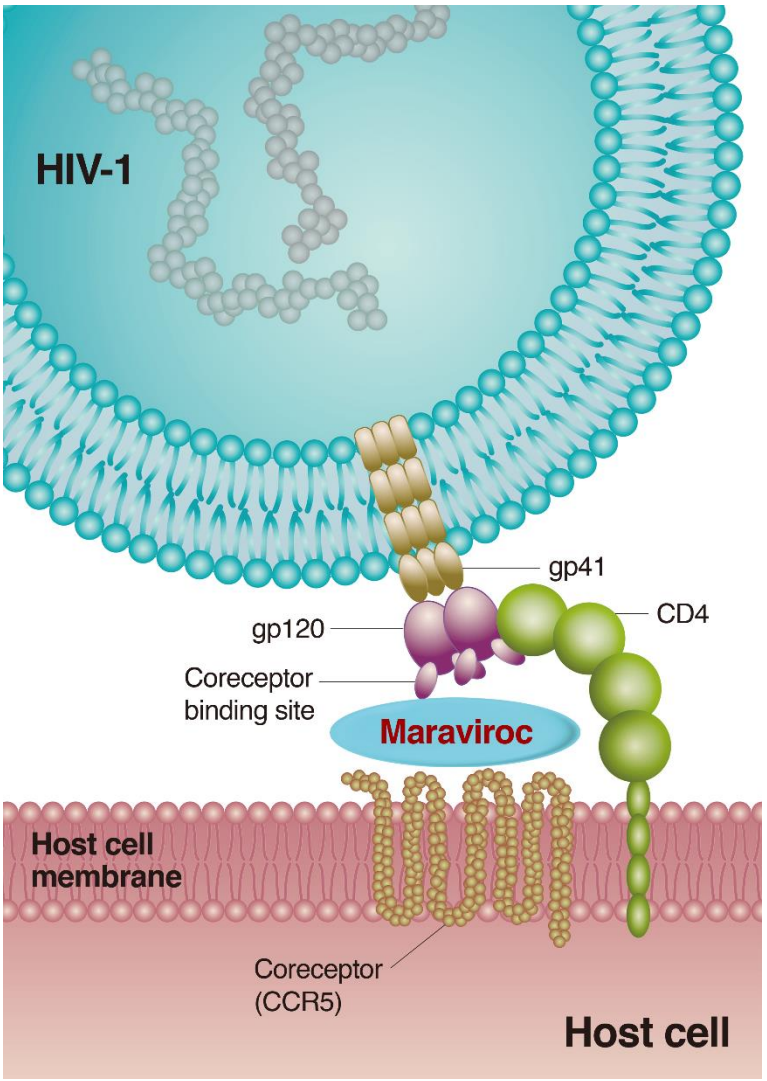

Figure 1. Drug action mechanism of maraviroc (CCR5 receptor antagonist).

Maraviroc selectively binds to CCR5 on the host cell membrane to prevent the virus from entering the host cell.

Phenotypic and genotypic assays can predict the coreceptor tropism of a patient's dominant virus population. Phenotypic assays use a patient-derived virus to test the susceptibility of the virus to specific CCR5 or CXCR4 inhibitors in vitro. This assay takes time to perform and requires a plasma HIV RNA level >1,000 copies/ml. Genotypic assays determine HIV-1 coreceptor usage based on the sequence of the V3 region of HIV-1 *env* which is the key determinant of coreceptor use. There are various bioinformatics programs used to predict coreceptor usage from the V3 sequence[5]. Genotypic assays are unable to completely differentiate X4 or dual-tropic variants, so phenotypic assays are recommended. Phenotypic assays show high specificity (~90%) but only modest sensitivity (~50% to 75%) for the presence of X4 virus[6]. However, taking into account the high accessibility, short turnaround time, and cost of genotyping, European guidelines currently include genotypic testing as an option which is equivalent to phenotypic testing for determining coreceptor usage in patients with HIV RNA >1,000 copies/ml and preferentially for those with HIV RNA ≤1,000 copies/ml[7].

Despite success in reducing HIV replication and improving the quality of life of many people living with HIV, due to the long-lived viral reservoir, cART does not eradicate the virus[8]. A reservoir is defined as 'a cell type or anatomical site in which a replication-competent form of virus accumulates and persists'[9,10]. HIV-1 persists as a stably integrated and replication-competent provirus in latent reservoirs[10]. Because of theses reservoirs, discontinuing cART can cause rapid rebound of viremia. Although resting memory $CD4^+$ T cells are best characterized as reservoirs, the proviral population can persist in other cells. Within an individual, HIV-1 can

vary between tissue types or cell types, thereby creating viral compartments. Several previous publications have reported that viral compartmentalization is also present between CSs[11-13]. HIV-1 has high mutability and capacity for rapid adaptation[14]. Quasispecies refers to mutant viruses that develop as a virus replicate within a host. HIV can mutate into multiple quasispecies, escaping the immune system's ability to suppress replication during HIV infection. Single genome sequencing can be used to analyze intra-patient polymorphisms. Theses polymorphic characteristics can be an important factor when deciding on a treatment drug for a patient.

The purpose of this study was to estimate the prevalence of CCR5 tropism among HIV-1 infected Koreans. Before the commercialization of the CCR5 receptor antagonist in Korea, it would be helpful to compare clinical factors according to coreceptor usage patterns. This study aimed to analyze the coreceptor tropisms of cells that can be used as reservoirs. In addition, the diversity and characteristics of polymorphisms possessed by each cell in an individual were investigated using single genome analysis.

II. MATERIALS AND METHODS

1. Prevalence of CCR5 tropism

A. Study design and population

HIV-1–infected patients older than 20 years were consecutively enrolled from four medical centers (Inha University Hospital, Korea University Guro Hospital, Kyungpook National University Hospital, and Severance Hospital) in three cities (Seoul, Degu, and Incheon) in South Korea between April 2013 and May 2014. Because the primary endpoint of this study was the prevalence of CCR-5 tropic HIV-1 infection, the sample size was calculated based on the previously reported prevalence of CCR5-tropic HIV-1. According to a study by Graeme *et al*, the proportion of HIV patients with CXCR4 or mixed/dual-tropic CCR5/CXCR4 was 19.9%[15]. Based on this report, the predicted rate of CCR5-tropic HIV-1 infected patients was 80% with 10% accuracy (95% length of confidential interval), the number of patients was 246 with a 95% confidential interval.

$$N = \frac{4Z_{\alpha}^{2}P(1-P)}{W^{2}}$$

Z_{α} = standard normal deviate for a two-sided α, where (1- α) is the confidence level (1.96)

P = expected proportion (0.8)

W = total width of confidence interval (0.1)

All patients provided informed consent for the collection and use of their blood sample and clinical data. This study was approved by the institutional review

board of each hospital (IRB#12-154, KUGH12201-001, KNUH2012-11-025, and 4-2012-0761), and the study protocol was posted on ClinicalTrials.gov.

B. Demographic and clinical data

Age, sex, date of HIV diagnosis, route of infection, HIV stage 3 classifications by the Center for Disease Control and Prevention (CDC), acquired immune deficiency syndrome (AIDS)-defining illness, history of pharmacological treatment (current antiretroviral therapy status – naïve or experienced, antiretroviral regimen), laboratory data including $CD4^+$ T cell and CD8+ T cell count (initial, pre-antiretroviral therapy (ART), nadir, peak and current), and HIV viral load and treatment resistance were collected and analyzed. The CDC classified HIV in three stage classification based on the degree of immune system damage (measured by the number of $CD4^+$ T cell) or presence of opportunistic infections. Category A is asymptomatic HIV infection without a history of symptoms or AIDS-defining conditions. Category B is HIV infection with symptoms that are directly attributable to HIV infection (or a defect in T-cell–mediated immunity) or are complicated by HIV infection. Category C is HIV infection with AIDS-defining opportunistic infections. The category of infection was defined at the time of diagnosis, and pre-ART was defined as the time immediately prior to treatment initiation. Nadir was defined as the lowest point of $CD4^+$ T cell count, and peak was defined as the highest point of HIV-1 viral load.

C. Genotype analysis

Coreceptor tropism of HIV-1 with HIV RNA or proviral DNA were identified by genotypic assay. Proviral DNA was used if the viral load of the patient was

<2000 copies/ml. HIV-1 RNA is transcribed into complementary DNA (cDNA), which serves as template. After two rounds of polymerase chain reaction (PCR), the V3 region of gp120 was amplified in abundance. These amplicons were then subjected to sequencing reaction and electrophoresis using an ABI Prism 3730xl DNA Sequencer (Applied Biosystems, Foster City, CA) and sequence data was collected. The standard population-based sequence was analyzed using sequence analysis software assigned, and exported in FASTA file format. The V3 genotyping assay involved sequencing the V3 loop of the gp120 protein which is highly correlated with its R5 or X4 tropism. The gp120 region spans the nucleotide sequence region (HXB2 position) 6225-7758 and the V3 loop spans the region 7205-7301 (Figure 2). Genotypic tropism was determined with web-based bioinformatics tool, Geno2pheno (http://coreceptor.geno2pheno.org). CCR5 tropism defined as having a false-positive rate (FPR) over 15%. HIV-1 subtype was determined by genome sequencing, which is the gold standard for subtype assignment.

D. Statistical analysis

Normally distributed continuous variables were expressed as mean ± standard deviation. Univariate logistic regression was used to assess differences between patients with and without CCR5 tropism in each variable. Variables with *P*-value < 0.20 on univariate logistic regression, and variables that were considered clinically meaningful (age, current $CD4^+$ T-cell count, and pretreatment viral load) were included for multivariate logistic regression analysis. The goodness-of-fit of the model was measured using the Akaike information criterion. Statistical analyses were conducted using SAS Software 9.4 (SAS Institute Inc., Cary, NC).

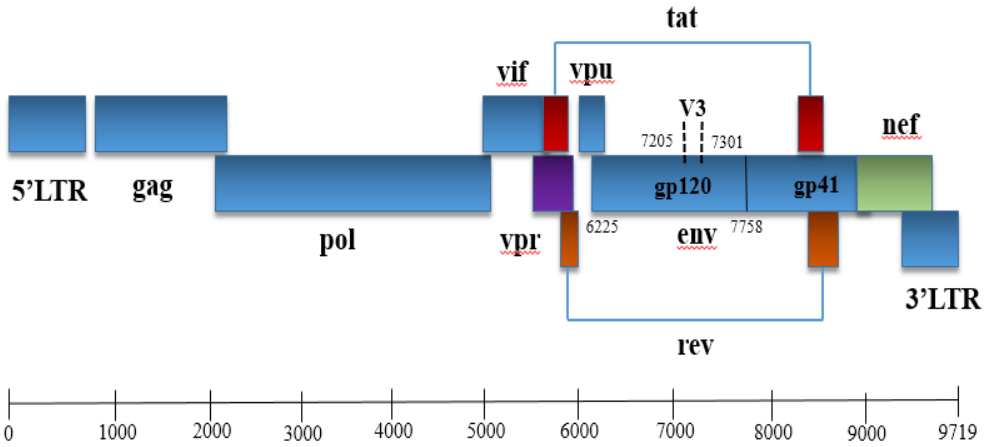

Figure 2. HIV-1 gene map. The gp120 region spans the nucleotide region (HXB2 position) 6225-7758 and the V3 loop spans the region 7205-7301.

LTR: long terminal repeat.

2. Genotypic tropism between the plasma and various CSs

A. Study design and population

Treatment-naïve HIV-1-infected patients were enrolled from Severance Hospital (a 2,000-bed university tertiary referral hospital in South Korea) between January and December 2012. The demographic and clinical characteristics of each subject were collected. All patients provided written informed consent. This study was approved by the IRB of Severance Hospital (IRB# 4-2011-0295).

B. Cell sorting

Samples (20 ml) of whole blood anticoagulated with EDTA were obtained from each patient by phlebotomy on day 1 of cART initiation and again six months later. Blood, plasma and peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque density gradient centrifugation (LymphoprepTM, Axis-Shield, Oslo, Norway). Naïve $CD4^+$ T cells, central memory and effector memory $CD4^+$ T cells, activated $CD4^+$ T cells, $CD14^+$ monocyte, and $CD56^+$ natural killer (NK) cells were sorted using a fluorescence-activated cell sorter (FACS) (Aria II, BD Bioscience, San Jose, CA). FITC-conjugated anti-CD3 monoclonal antibodies (mAbs, BioLegend, San Diego, CA) were added to label CD3 cells in the PBMC. Positively selected CD3 cells were then immediately stained with PE-conjugated anti-CD4 (BioLegend, San Diego, CA), APC-conjugated anti-CD45RA (BioLegend, San Diego, CA), perCP/Cy5.5-conjugated anti-CCR7 (BioLegend, San Diego, CA), and APC/Cy7-conjugated anti HLA-DR mAbs (BioLegend, San Diego, CA). Negatively selected CD3 cells were stained with perCP/Cy5.5-conjugated anti-CD14 (BioLegend, San Diego, CA) and APC/Cy7-conjugated anti-CD56 mAbs (BioLegend, San Diego, CA). Cells were sorted by flow

cytometry on a FACS. Cell purity was determined by flow cytometry analysis of the sorted cells on the same instrument with the same instrument settings. The sorted cell populations were routinely >90% pure.

$CD3^+/CD4^+/HLA\ DR^-/CD45RA^+/CCR7^+$ cells were considered resting naïve $CD4^+$ T cells. $CD3^+/CD4^+/HLA\ DR^-/CD45RA^-/CCR7^+$ cells were considered resting central memory $CD4^+$ T cells. $CD3^+/CD4^+/HLA\ DR^-/CD45RA^-/CCR7^-$ cells were considered resting effector memory $CD4^+$ T cells. $CD3^+/CD4^+/HLA\ DR^+$ cells were considered activated $CD4^+$ T cells. $CD3^-/CD14^+$ cells were considered $CD14^+$ monocytes. $CD3^-/CD56^+$ cells were considered NK cells.

C. Single genome amplification and genotype analysis

HIV RNA was extracted from plasma using the QIAmp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and reverse-transcribed into cDNA with the RETROscript kit (Applied Biosystems, Foster City, CA). Genomic DNA from each cellular compartment was isolated using the QIAmp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. For single genome amplification and sequencing, nested PCR of *env* C2V3 was performed using 10 μl of diluted cDNA or cellular DNA template added to 40 μl of reaction mixture for the first round. The reaction mixture consisted of 5.0 μl of 10× PCR buffer containing magnesium chloride and 1.0 μl of 10 nM dNTP Mix (GeneAmp, Applied Biosystems), 0.25 μl of Taq DNA Polymerase (Roche Diagnostics, Indianapolis, IN), 31.75 μl of molecular grade water, and 1 μl each of two 20 μM primers, V3-forward primer (5'-CAAAGGTATCCTTTGAGCCAAT-3') and V3-reverse primer (5'-ATTACAGTAGAAAAATTCCCCT-3'). The 50 μl samples were heated to 95℃ for two min and then subjected to 35 cycles of 30s at 95℃

followed by 30s at 50℃ followed by 60s at 72℃. The samples were heated to 72℃ for 10 min and then kept at 4℃ until use. Second round PCR used 5 μl of the first-round product as the template added to 45 μl of reaction mixture for a total volume of 50 μl. This reaction mixture consisted of the reagents, but the volume of the molecular grade water was increased to 36.75 μl. The primers were V3-forward primer (5'-GAACAGGACCAGGATCCAATGTCAGCACAGTACAAT-3') and V3-reverse primer (5'-GCGTTAAAGCTTCTGGGTCCCCTCCTGAG-3'). The thermal cycling parameters were the same as for the first round. Twenty PCR products from each sample were sequenced using Prism Dye terminator kits on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Sequences were initially edited and aligned by bioinformatics analyses using Clustal W[16,17]. Alignment was manually edited in Bioedit, version 7.05 to preserve frame insertions and deletions if present. The sequences of each patient was been submitted to GenBank (Genbank accession number: KP796426-KP797835)

Genotypic tropism was determined with a web-based bioinformatics tool, Geno2pheno (http://coreceptor.geno2pheno.org). CCR5 tropism was defined as having an FPR over 10%.

III. RESULTS

1. Prevalence of CCR5 tropism

A multicenter, cross-sectional observational study was conducted to determine the prevalence of CCR5-tropic HIV-1 infection and the factors associated with CCR5 tropism in Korea. In total, 250 patients with HIV were enrolled, but only 143 patients were analyzed for genotypic tropism assay with HIV RNA or proviral DNA. Proviral DNA was used if the viral load was under 2,000 copies/ml. There were only two samples with a viral load above 2,000 copies/ml. One hundred forty-two samples (98.6%) had a viral load of under 2000 copies/ml and were underwent proviral DNA testing.

The prevalence of CCR5 tropism was 69.2% (99/143). The major subtype (or 'clade') of HIV-1 was subtype B (Figure 3) which accounted for 83.8% (83/99) in the CCR5 group and 88.6% (39/44) in the non-CCR5 group. Of the remaining patients in the CCR5 group, 11 were subtype C, two were subtype A or AG, two were subtype G, and one was subtype F. In the non-CCR5 group (X4 or R5/X4), two patients were subtype C, two were subtype AE, and one was subtype A or AG. The majority of patients in both groups were male (CCR5 group, 94%, 93/99; non-CCR5 group, 93.2%, 41/44). The major routes of HIV exposure were sexual contact; with 32.3% (32/99) and 25% (11/44) of cases transmitted by men who had sex with men in the CCR5 and non-CCR5 groups, respectively; although the number of cases of transmission was slightly higher in the CCR5 group, there was no statistical significance. Heterosexually transmitted cases accounted for 29.3% (29/99) and 40.9% (18/44) in CCR5 and non-CCR5 groups, respectively. The HIV disease period was higher in the CCR5 group (66.66±45.56 months) than in the non-CCR5 group (50.87±45.44 months) (Table 1).

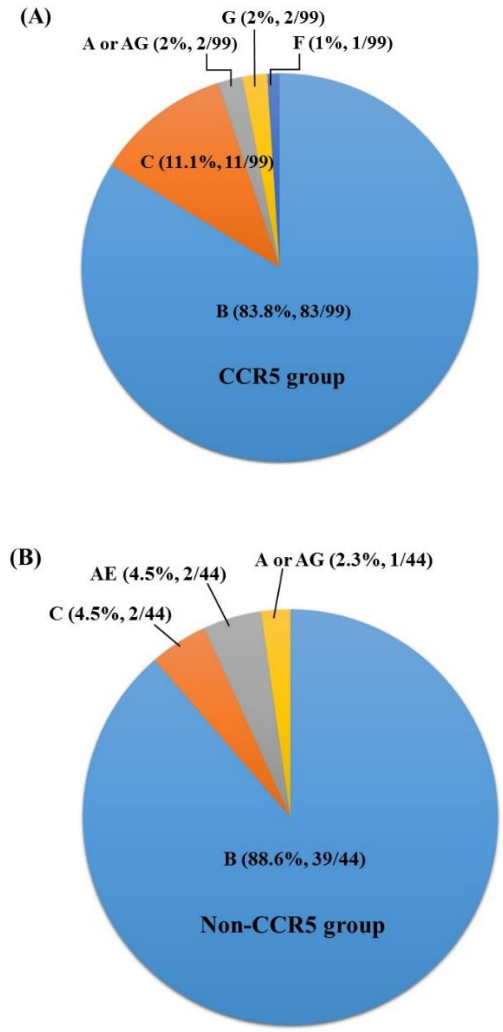

Figure 3. HIV-1 subtype analysis in CCR5 and non-CCR5 groups. The major subtype of HIV-1 was B in both groups.

Table 1. Clinical characteristics of CCR5 and non-CCR5 patients (n=143)

Variable	CCR5 (n=99, 69.2%)	Non-CCR5 (n=44, 30.8%)	P-value
Age, years	45.05±12.46	44.77±11.65	0.839
Sex			
Male	93 (94.0)	41 (93.2)	0.863
Female	6 (6.1)	3 (6.8)	0.493
HIV exposure (overlapped)			
Homosexual contact	32 (32.3)	11 (25.0)	0.485
Heterosexual contact	29 (29.3)	18 (40.9)	0.293
Intravenous drug use	0 (0.0)	0 (0.0)	
Receipt of blood/products	0 (0.0)	1 (2.3)	0.988
Perinatal transmission	0 (0.0)	0 (0.0)	
Other	45 (45.5)	18 (40.9)	0.769
CDC classification			
A[1]	64 (66.7)	24 (65.7)	0.868
B[2]	5 (5.21)	3 (8.6)	0.476
C[3]	27 (28.1)	9 (25.7)	
HIV disease period, months	66.66±45.56	50.87±45.44	

Values are given as n (%) or mean ± standard deviation.

[1] A: asymptomatic HIV infection.

[2] B, HIV with symptoms that are directly attributable to HIV infection.

[3] C: HIV infection with acquired immune deficiency syndrome-defining opportunistic infections.

CDC: Centers for Disease Control and Prevention.

Of the 143 patients with HIV, only two in the CCR5 group (2.02%, 2/99) were treatment-naïve. The initial cART regimen was mostly protease inhibitor (PI)-based ART (Table 2). There was no patient using maraviroc. The $CD4^+$ T cell count and viral load of CCR5 and non-CCR5 subjects during the initial, pre-ART, nadir, and current treatment periods were not different (Table 3). There were no statistically significant clinical or epidemiological predictors for CCR5 tropism among the enrolled patients.

2. Genotypic tropism between the plasma and various cellular subsets

A. Patients and sample characteristics

Five treatment-naïve patients with HIV-1 were enrolled. Demographic and clinical characteristics of the enrolled subjects are shown in Table 4. HIV RNA and DNA populations were sequenced from blood plasma and CSs from all five patients immediately before and six months after cART initiation. All five patients were male, and the average age was 31.8 years. The subtypes of HIV were B in all patients. Six months after commencing cART treatment regimen, the viral loads were suppressed to <20 copies/ml in three patients and <200 copies/ml in two patients. $CD4^+$ T cell counts were lowest in Patient B at baseline and six months after cART initiation.

Table 2. Medication history of CCR5 and non-CCR5 patients

Variables	CCR5 (n=99, 69.2%)	Non-CCR5 (n=44, 30.8%)	Total (n=143)
Experienced on treatment	97 (97.98)	44 (100)	141 (98.6)
Naïve	2 (2.02)	0 (0)	2 (1.4)
Initial ART			
NNRTI-based cART	23 (23.23)	14 (31.82)	37 (25.87)
PI-based cART	71 (71.72)	26 (59.09)	97 (67.83)
Other	5 (5.05)	4 (9.09)	9 (6.29)

Values are given as n (%).
ART: antiretroviral therapy, NNRTI: non-nucleoside reverse transcriptase inhibitor, cART: combination antiretroviral therapy, PI: protease inhibitor.

Table 3. $CD4^+$ T cell count and HIV-1 viral load of CCR5 and non-CCR5 patients (n=143)

Variable	CCR5 (n=99, 69.2%)	Non-CCR5 (n=44, 30.8%)	P-value
$CD4^+$ T cell count (cells/μl)			
Initial test[1]	190.5 (64.0, 340.0)	194.0 (67.0, 358.0)	0.9448
Pre-ART test[2]	157.0 (55.0, 285.0)	118.0 (47.0, 244.0)	0.9083
Nadir[3]	110.0 (44.0, 276.0)	101.5 (33.0, 225.5)	0.7081
Current	473.0 (295.0, 667.0)	482.5 (312.0, 688.5)	0.4707
HIV-1 viral load (copies/ml)			
Initial test	111,000.0 (23,000.0, 430,000.0)	86,150.0 (33,934.0, 312,000.0)	0.9980
Pre-ART test	118,000.0 (34,400.0, 382,523.5)	160,000.0 (54,000.0, 315,318.0)	0.7057
Peak[4]	107,00.0 (11,358.0, 361,000.0)	12,494.5 (34,800.0, 307,659.0)	0.5335
Current	19.0 (0.0, 23.0)	19.0 (19.0, 20.5)	

Values are given as median (IQR Q1, Q3).
[1] At the time of diagnosis.
[2] At the time immediately before starting treatment.
[3] The lowest point of $CD4^+$ T cell coun.t
[4] The highest point of HIV-1 viral load.

Table 4. Patients and HIV sequencing characteristics

				Plasma HIV RNA (copy/ml)		$CD4^+$ T cell count (cells/ml)	
Patient	Sex	Age (years)	HIV subtype	Baseline[1]	6 months[2]	Baseline	6 months
A	Male	26	B	61,800	<20	176	361
B	Male	25	B	3,150,000	141	84	183
C	Male	51	B	85,300	58	244	703
D	Male	26	B	14,300	<20	324	323
E	Male	31	B	12,100	<20	453	531

[1] Immediately before treatment initiation.
[2] Six months after treatment initiation.

B. Tropism assay between the plasma and various cellular subsets before and after treatment

Corecptor tropism analysis was performed via single genome sequencing in plasma for each CS before and after treatment initiation (Table 5). A mean of 282 C2V3 *env* sequences (range: 277-292) was obtained per patient, and about 20 sequences per plasma and CS (range: 17-24) were identified. Two of the five patients (Patients A and D) had X4 strains in both plasma and CSs and the patterns were the same before and six months after cART initiation. In the other three patients, the tropisms of plasma and CSs were discordant before and six months after cART initiation. In Patient B, the heterogeneous population was monocyte. In Patient E, naïve $CD4^+$ T cells and NK cells had more genetic diversity. Patients B and E had X4 strains in plasma and most CSs. However, in Patient B, only R5 strains were observed in monocytes before starting cART. In Patient E, naïve $CD4^+$ T cells and NK cells were observed. In Patient C, more genetic diversity was observed after six months of treatment; before treatment initiation, most of the strains were X4 and R5 strains were detected in NK cells only, but after treatment, R5 strains were detected in almost all CSs except for naïve $CD4^+$ T cells.

Table 5. Characteristics of HIV tropism in five patients in plasma samples and cellular subsets

	Number of sequences at pretreatment			Number of sequences at post-treatment		
Patient	Cellular subset	CCR5	CXCR4	Cellular subset	CCR5	CXCR4
A	TCA	0	20	TCA	0	20
	TCM	0	20	TCM	0	20
	TEM	0	20	TEM	0	20
	Monocytes	0	20	Monocytes	0	20
	Naïve $CD4^+$ T cells	0	20	Naïve $CD4^+$ T cells	0	19
	NK cells	0	20	NK cells	0	19
	Plasma	0	20	Plasma	0	20
B	TCA	0	22	TCA	0	19
	TCM	0	20	TCM	0	21
	TEM	0	21	TEM	0	20
	Monocytes	5 (26.3)	14 (73.7)	Monocytes	0	20
	Naïve $CD4^+$ T cells	0	22	Naïve $CD4^+$ T cells	0	18
	NK cells	0	20	NK cells	0	20
	Plasma	0	22	Plasma	0	18
C	TCA	0	24	TCA	8 (38.1)	13 (61.9)
	TCM	0	21	TCM	12 (60.0)	8 (40.0)
	TEM	0	21	TEM	2 (9.5)	19 (90.5)
	Monocytes	0	24	Monocytes	6 (33.3)	12 (66.7)
	Naïve $CD4^+$ T cells	0	22	Naïve $CD4^+$ T cells	0	22
	NK cells	15 (78.9)	4 (21.1)	NK cells	3 (15.0)	17 (75.0)
	Plasma	0	20	Plasma	0	19
D	TCA	0	20	TCA	0	20
	TCM	0	20	TCM	0	20
	TEM	0	19	TEM	0	20
	Monocytes	0	19	Monocytes	0	20
	Naïve $CD4^+$ T cells	0	20	Naïve $CD4^+$ T cells	0	20
	NK cells	0	20	NK cells	0	20
	Plasma	0	19	Plasma	0	20
E	TCA	0	20	TCA	0	20
	TCM	0	21	TCM	0	20
	TEM	0	20	TEM	0	20
	Monocytes	0	20	Monocytes	0	20
	Naïve $CD4^+$ T cells	2 (10.0)	18 (90.0)	Naïve $CD4^+$ T cells	0	20
	NK cells	6 (30.0)	14 (70.0)	NK cells	0	20
	Plasma	0	20	Plasma	0	19

TCA: Activated $CD4^+$ T cells, TEM: Effector Memory $CD4^+$ T cells, TCM: central memory $CD4^+$ T cells, NK: natural killer

IV. DISCUSSION

1. Prevalence of CCR5 tropism

Therapeutic decisions are usually based on plasma viremia and $CD4^+$ T cell count parameters. However, the current development of CCR5 and CXCR4 antagonists may compel the modification of this paradigm[18]. This study aimed to determine the prevalence of CCR5 tropism among HIV-1–infected patients in South Korea. The prevalence of CCR5 and CXCR4 tropisms is important information for evaluating the potential use of CCR5 antagonists. CCR5 receptor antagonists target a host coreceptor to inhibit HIV entry into $CD4^+$ T cells and monocytes/macrophages[4]. Based on phase 3 clinical studies demonstrating its safety and efficacy, Maraviroc was approved by the U.S. Food and Drug Administration in 2007 for the treatment of HIV infection in individuals "with CCR5-tropic HIV-1 only"[19]. Unlike other antiretroviral drugs, maraviroc targets infection in individuals through antagonism of the coreceptor rather than the virus itself. Thus, maraviroc has been produce therapeutic activity in patients who are resistant to other antiretroviral drugs.

In this study, the prevalence of CCR5 was 69.2%. Bon *et al.* performed genotypic assays in samples from highly treatment-experienced patients and found a high prevalence (>70%) of R5 strains[20]. Ferrer *et al*. conducted a prevalence study in 454 patients who needed to change their ART regimen due to virologic failure[21]. Virologic failure is defined by a lack of a complete viral load suppression (<20 copies/ml). According to their study, 66% (299/454) of patients had virus with tropism, 22% (102/454) samples had X4 tropism, and 12% (53/454) were non-

reported. The prevalence of R5 tropism in the current study was similar to that shown in both previous studies. However, the target patient group in the other studies were unable to proceed with conventional ART due to virologic failure, whereas the current patient group included HIV patients only.

Geno2pheno, a web-based bioinformatics tool, predicts HIV-1 coreceptor usage from the V3 region of the HIV envelope protein gp120. In the current study, FPR was set at 15%. This means that an FPR value under 15% was predicted for X4 virus, while an FPR value equal to or above 15% indicated R5 virus. A Geno2pheno cutoff FPR of 5.75% has been shown to be a good predictor of sustained response in several clinical trial datasets. However, there was no fixed FPR for users. Usually, an FPR between 10% and 20% is recommended, based on plasma HIV RNA loads[22]. In the current study, an FPR of 15% was selected to ensure high sensitivity in detecting X4 or R5/X4 variants. CCR5 prevalence was 69.2% (99/143) for 15% FPR, 72.0% (103/143) for 10% FPR, and 80.4% (115/143) for 5% FPR. Parczewski *et al.* performed a cohort study of 194 patients with newly diagnosed HIV-1 genotypic tropism using triplicate V3-loop sequencing[23]. They found that the overall R5 tropism frequency for a 5.75% FPR was 84.53% (164/194), and 72.16% (140/194) for a 10% FPR.

Bader *et al*. investigated whether tropisms could be used as a parameter for disease progression[24]. They reported that 60% (9/15) of patients with X4-tropic viruses and 46.4% (32/69) of patients with R5-tropic viruses were incomplete responders (p=0.339) whose $CD4^+$ T cell count remained <400 cells/μl after five years on cART. In another study, Waters *et al.* investigated 402 treatment-naïve patients, and found that the presence of dual/mixed or X4-tropic virus has a deleterious effect on $CD4^+$ T cell count decrease and risk of clinical disease[25]. In

the current study, the median $CD4^+$ T cell count on nadir was 110 (44.0-276.0) in the CCR5 group and 101 (33.0-225.5) in the non-CCR5 group; and the current median $CD4^+$ T cell count was 473 (295.0 to 667.0) in the CCR5 group and 482.5 (312.0 to 688.5) in the non-CCR5 group (Table 3). Although not statistically significant, the median $CD4^+$ T cell count on nadir was slightly lower in the non-CCR5 group which is probably the X4-tropic or dual-tropic group. However, the participants had different treatment histories, making it difficult to give meaning.

In the current study, HIV-1 subtype was characterized and compared between CCR5 and non-CCR5 groups. HIV-1 subtypes are phylogenetically linked strains of HIV-1 that are approximately the same genetic distance from one another.[26]. Currently known HIV-1 subtypes are subtypes A, B, C, D, F, G, H, J, and K. Of note, A through D are highly prevalent. Advances in full-genome sequencing technology have made it possible to identify circulating and unique recombinant forms. CRF01_AE and CRF02_AG are found principally in Southeast Asia and West Africa, respectively. The other subtypes have more limited distributions. Subtype B which is widely distributed in Americas, Western Europe, East Asia, and Oceania was the main subtype found in the subjects in the current study. Subtype B usually uses CCR5 early, with increasing use of CXCR4 in late infection[27]. The second most common subtype found in the current study was subtype C, which is distributed primarily in India and Eastern and Southern Africa. Subtype C mainly uses CCR5 coreceptors. Both subtypes B and C mainly use CCR5. However, while subtype B changes to use CXCR4 in the later stages of disease, subtype C continues to use CCR5 throughout the course of infection.

Clinical data, including age, gender, date of diagnosis, route of infection, CDC classification, AIDS-defining illness, and treatment history were collected and

compared between groups, without significant differences. As expected, there were no differences in $CD4^+$ T cell counts between groups, as the baseline and current $CD4^+$ T cell counts in the study cohort were very low.

2. Genotypic tropism between the plasma and various cellular subsets

The 'shock and kill' method is a famous model which is currently used in the search for a cure for HIV-1 infection. Although cART is very effective in controlling viremia, the virus remains hidden in the immune system due to the presence of reservoirs[28]. The main reservoir is the central memory $CD4^+$ T cell[29]. However, proviral DNA populations are also found in naïve $CD4^+$ T cells, tissue macrophages, peripheral blood monocytes, and NK cells during cART[30-33]. To advance the search for a cure, more detailed information about viral reservoirs is essential. This study aimed to further our understanding about the cellular source of HIV RNA and the differences before and after cART. HIV populations in blood plasma and six different CSs were sampled and sequenced before and after six months of cART. HIV-1 DNA was detected from six cellular subsets at two points.

HIV-1 coreceptor usage is an important determinant of progression in the natural history of the disease[34]. R5 viruses are predominate during early infection, while X4 viruses are rarely seen[35-37]. X4 viruses are seen in approximately 40~50% of patients during the late disease progression[35]. One French study in treatment naïve patients demonstrated that 15.9% (62/390) of patients harbored X4 or dual-tropic viruses in PBMCs at the time of primary infection[38]. In the first part of the current study, the prevalence of CCR5 tropism was 69.2%; but in the second part of the study, samples showed a form of X4 variants. One possible reason for these findings is that the patients were treatment-naïve in the second part of the study.

However, it is probable that the disease was in the chronic stage of disease progression. In patients whose disease had progressed to the chronic stage, tropisms were not different from previous studies. However, because the sample size was very small, it is difficult to give meaning to the results.

In the current study, three of the five patients (Patients B, C, and E) showed discordant tropism patterns. Parisi *et al*. reported 10% (10/100) discordant tropism between plasma and PBMCs in treatment naïve patients[38], four patients had R5 virus in plasma and X4 virus in PBMC and six patients had X4 virus in plasma and R5 virus in PBMCs. However, the differences in coreceptor tropism between before and after treatment were not shown. Raymond *et al*. in another study, reported that HIV-1 tropisms in the plasma and PBMCs from patients in the primary stage of infection were 98% concordant[39]. In the current study, monocytes from Patient B, and naïve $CD4^+$ T cells and NK cells from Patient E showed R5 virus before cART but all changed to X4 virus after treatment. Unusually, Patient C showed an increased proportion of R5 virus after cART. All five patients' plasma samples showed R5. The presence of X4 virus in plasma and R5 virus in other CSs may represent a switch of viral tropism early during infection progression. If a virus variant using CXCR4 appears first, it does not immediately dominate the virus population in any compartment[40]. Instead, the virus can fluctuate over time at levels near the detection limits of assays. Tropism discordances in different CSs are likely due to low-level minority species present as quasispecies that are undetected by bulk sequencing. In one report, cellular quasispecies were found to be more heterogeneous than those observed in plasma[41]. However, the tropism discordance shown in Patient C, in the current study, is only speculative.

This study has several limitations. The prevalence study had a cross-sectional design in which treatment naïve patients and cART-treated patients were mixed. Possible bias derived from a high rate of PCR failure is another limitation of this study. Because only 143 of the 250 samples had identifiable tropism, the results may not be generalizable within Korea. The non-CCR5 group was estimated to have X4-tropic or dual/mixed viruses however, it is not exactly known because only the genotypic method of testing. In the second part of study, the difference in tropisms before and after cART is meaningful. However, an inability to accurately analyze the cause of these result due to the small sample size is also limitation of this study.

V. CONCLUSION

The prevalence of CCR5 tropism in Korean HIV-1 infected individuals was 69.2%. Because coreceptor tropisms could not be predicted by clinical factors, the tropism assay should be conducted before treatment with a CCR5 antagonist. Results demonstrated that coreceptor usage was discordant between cellular subsets, and before and after six months of treatment. Tropism discordance between plasma and CSs is relevant because it may influence clinical decisions regarding the use of CCR5 antagonists in HIV-1-infected patients. Further studies with larger cohorts and longer tracking periods are required.

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ABSTRACT (IN KOREAN)

한국 HIV-1 감염자에서 혈액과 세포 아집단 내 HIV-1 보조수용체 친화성의 특징

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송제은

사람면역결핍바이러스(HIV)는 감염을 일으켰을 때 환자에게서 면역장애를 점진적으로 유발하는 바이러스이다. 바이러스가 숙주를 감염시키기 위해서 세포에 들어갈 때 보조수용체가 필요한데, 대표적으로 CCR5과 CXCR4가 있다. 이 두 가지 보조수용체를 발견함으로 바이러스와 숙주 세포 사이의 상호 작용에 대한 이해가 향상되었고 새로운 치료법 개발이 발달하게 되었다. 특히 CCR5 길항제가 개발되어 HIV 치료에 희망으로 여겨진다. 하지만 바이러스는 저장소가 있어 혈중의 바이러스를 박멸하더라도 조직, 세포에 남아있어 HIV-1은 아직은 완치가 불가능한 질환이다. 또한, HIV-1은 변이를 잘 일으키기 때문에 개인에 있어서도 다양한 모습을 보이게 되고, 이로 인해 약물 내성 등의 문제도 제기된다. 이 연구에서는 CCR5 길항제의 국내 상용화 전에 국내 감염인의 보조수용체 친화성의 유병률 및 임상양상의 특성을 알아보고자 한다. 또한 개인 내에서 저장소로 사용될 수 있는 세포들을 비교분석하고, 세포 내에서 보조수용체 친화성이 다형성을 보이는지 알아보고자 한다.

첫 번째로, 2013년 4월부터 2014년 5월까지 3개 도시의 4개 의료센터에서 HIV-1에 감염된 한국인을 등록했다. 바이러스 외피 단백의 V3 영역에 대한 중첩된 중합효소 연쇄반응 및 집단-기반 염기서열결정을 HIV RNA 또는 proviral DNA로 수행하였다. HIV-1의 보조수용체 친화성은 웹 기반 생물정보학도구인 geno2pheno

(http://coreceptor.geno2pheno.org)를 사용하여 결정하였다. 두 번째로, 2012년 1월부터 12월까지 새로 치료를 시작하는 HIV 감염인을 대상으로 항레트로바이러스 치료 개시일 및 치료 개시 6개월 후 혈액을 채취하였다. 형광표지세포분류기를 사용하여 Naïve $CD4^+$ T 세포, 휴식기 중심 기억 그리고 효과 기억 $CD4^+$ T 세포, 활성화 $CD4^+$ T 세포, 단핵구, 그리고 자연살해세포를 분류하였다. 그리고 혈액 내 HIV RNA 또는 proviral DNA를 이용하여 바이러스 외피 단백의 C2V3 영역에 대한 중첩된 중합효소 연쇄반응 및 염기서열결정으로 분석을 시행하였다. 보조수용체 친화성 식별은 geno2pheno를 이용하였다.

첫 번째 연구에서, 250명이 연구에 등록되었고 그 중에 143명의 감염인이 분석대상이 되었다. CCR5 보조수용체의 비율은 69.2%였고, 임상적 특징을 비교했을 때 비-CCR5 군과 유의미한 임상적 예측인자는 없었다. 두 번째 연구에서는, 치료력이 없는 5명의 감염인이 등록되었고, 혈장 및 세포 아집단 사이의 보조수용체 사용양상을 분석하였다. 5명 중 3명의 환자에게서 각 세포 아집단 내에서 보조수용체 사용양상이 항레트로바이러스 치료 전후하여 변화하는 양상을 보였다.

한국의 HIV-1 감염인에서 CCR5 보조수용체 친화성의 유병률은 이전 연구들과 비교해서 큰 차이는 없었다. 비-CCR5 군과 임상인자의 유의한 차이는 없었으므로 임상적 특징으로 보조수용체 사용을 예측하기는 힘들다. 따라서 CCR5 길항제를 사용하기 전, 반드시 보조수용체 친화성 식별이 진행되어야 한다. 또한, 혈장의 다양한 세포들이 바이러스의 저장소로 중요하게 작용을 하고 있으며, 한 환자 내에서도 다형성을 보이며 치료 전후에 불일치하는 양상을 나타낸다는 것을 알 수 있었다. 다만 두 번째 연구의 결과에 대해서는 아직 알려져있는 것이 많지 않으며 본 연구에서는 대상환자수가 적었다는 제한점이 있으므로 앞으로 더 많은 환자를 등록하고 더 오랜 기간에 걸쳐 추적 관찰 하는 추가 연구가 필요할 것이다.

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PUBLICATION LIST

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