Infectivity of Homologous Recombinant HIV-1 Pseudo-virus with Reverse Transcriptase Inhibitor-related Mutations from Highly Active Antiretroviral Therapy Experienced Patients

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Abstract

Objectives: In this study, the viral fitness of pseudo-viruses with a drug-resistant site in the reverse transcriptase (RT) region of the genome was investigated. The pseudo-viruses were derived from highly active antiretroviral therapy (HAART)-experienced HIV/AIDS patients.

Methods: HIV-1 RNA was extracted from the plasma of HAART-experienced (KRB9149, KRB7021, KRC1097) and HAART-naive (KRC5180, KRC5123) HIV-1 patients. The RT gene from the extracted viral RNA was amplified and the polymerase chain reaction product was cloned from the pHXB2_D2-261 RT vector. C8166 and TZM-bl cell lines were used as the HIV-1 replication capacity measurement system. To quantify the infectivity of homologous recombinant HIV-1, the infectivity derived from each pseudo-virus was compared with the infectivity of the reference strain HXB2.

Results: Patient-derived HIV-1 was cotransfected into C8166 cells and the expression level of the p24 antigen was measured. The expression was high in the HIV-1 isolates from patients KRC5180 and KRB9149 and low in patients KRB7021, KRC5123, and KRC1097, when compared with the reference strain. The infectivity of the pseudo-virus measured in TZM-bl cells decreased in the order, reference strain HXB2 > KRC5180 > KRC5123 > KRC1097 > KRB9149 > KRB7021 > KRC1097.

Conclusion: In this study, HIV-1 infectivity of the drug-resistant strain isolated from HAART-experienced patients with HIV/AIDS was found to be lower than the infectivity of the reference strain HXB2. This study provides useful data for the phenotypic susceptibility assay in HAART-experienced patients infected with HIV-1.
1. Introduction

The genetic variations of HIV-1 evolved in unique directions influenced by antiretroviral drugs, and the genetic or immunological characteristics of the host [1]. Therefore, it is expected that the virus clone adapted to the environment may be evolved and disseminated [2,3].

Antiretroviral drugs are one of the significant selection pressures that induce HIV-1 genetic variations. Since the introduction of combination therapy in the mid-1990s, antiretroviral drugs have contributed to the decreased mortality of HIV/AIDS patients by controlling disease progression and extending their lives [4]. However, antiretroviral drug-resistant variants of the virus have appeared among HIV/AIDS patients on highly active antiretroviral therapy (HAART). These drug-resistant variants also exhibit cross-resistance to similar and sometimes different kinds of therapeutic drugs. The control of drug resistance, therefore, has become an important issue, not only in HAART-experienced patients, but also in patients where treatment has failed [5].

The prevalence of drug resistance of HAART-experienced HIV/AIDS patients referred to the Korea National Institute for Health for genotypic drug resistance tests was 20–30% between 2006 and 2007 in Korea [6]. After its introduction in 1991, AZT (Zidovudine) has become a widely used drug in Korea [7]. A national survey showed that 20% of HAART-experienced HIV/AIDS patients were resistant to AZT. Patients with variants of the virus with D67N, T215Y or K219Q mutations in the protein coded by the thymidine-related drug-resistant gene, were found to be amongst those that were most resistant [6]. Variants with dual resistance against the protease (PR) and reverse transcriptase (RT) regions have also appeared [6]. Therefore, it is necessary to study the drug resistance and infectivity of HIV-1 variants, to better understand the factors that affect their dissemination.

In Korea, the drug resistance of clinical isolates has been investigated by genotypic assay according to drug treatment, viral load, and CD4 T cell counts [6]. However, these data are limited in their ability to analyze some of the important factors for drug resistance. By measuring HIV-1 p24 antigen production in HIV-1 infected cells or by measuring HIV-1 infectivity in cells, the replication capacity of HIV-1 variants was estimated and used to analyze drug resistance in the variants. Silliciano et al reported various protocols that can be used to measure the infectivity or replication capacity in vitro by infecting the recombinant clones with the whole or special region of the resistant gene from clinical isolates [8,9].

In this study, the viral fitness of pseudo-viruses derived from clinical isolates with drug-resistant sites in the RT region of the HIV-1 genome in HAART-experienced HIV/AIDS patients was investigated.

2. Materials and Methods

2.1. Recombinant vector construction and cotransfection

C8166 and TZM-bl cell lines were selected for the HIV-1 replication capacity measurement system. The pHXB2Δ2-261 RT vector (Stanford University, CA, USA) was cultured using the TOPO TA cloning kit (Invitrogen, CA, USA) and DNA was extracted using the HiSpeed™ Plasmid Midi Kit (Qiagen, CA, USA). Transformation was confirmed after treating with the restriction enzyme SmaI. pHXB2Δ2-261 RT and the target gene were transfected into C8166 cells with Lipofectamine 2000 (Invitrogen) and the cells were cultured at 37°C in 5% CO₂. To get a stable recombinant cell line, after 24 hours of transfection, the culture was scaled up to 6-wells by dilution with 1:10 growth medium. After 7 days, the culture medium containing the patient-derived virus infected cells was collected and centrifuged at 400 g for 10 minutes. Supernatants of the C8166 cultured medium were filtrated with 0.2 mm disposable syringe filters (Nalgene, Rochester, NY, USA). HIV-1 isolates were cotransfected with pHXB2Δ2-261 RT. The HIV-1 p24 antigen levels were measured twice using the supernatants that had been cultured for 7–8 days after the infection.

2.2. HIV-1 RNA quantitation and polymerization of HIV-1 pol gene

After extracting the RNA from the plasma using the NucliSens Extractor (BioMerieux, Boxtel, The Netherlands), viral load in blood was measured using nucleic acid sequence-based amplification. The reverse transcription polymerase chain reaction (RT-PCR) and PCR conditions were based on the Stanford Center for AIDS Research laboratory protocol for sequencing the PR and RT parts of pol. RT-PCR and nested PCR were performed as described earlier [7].

2.3. Analysis of predicted drug resistance

Sequencing of the HIV-1 pol gene was performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, MA, USA). The sequences were analyzed with the Stanford HIV-SEQ program, a HIV-1 drug resistance interpretation algorithm, and the HIV-1 RT and PR sequence database to focus on drug resistance-related mutation sites.

2.4. Infectivity measurement using TZM-bl cell line

Viral supernatants (10⁻¹, 1, 2, 5, 10, 20, 50, 100 μL) were added to 24 hours pre-cultured cell with a concentration of 10⁴ cells/100 μL. The virus-exposed
| Strain or patient | Subtype | Drug resistance-related polymorphic sites\(^a\)/Drug resistance interpretation |
|------------------|---------|--------------------------------------------------------------------------------|
| Reference (HXB2-IIIb) | B       | None/ None/ None/ None/ Wild type |
|                   |         | ATV(S), DRV(S), FPV(S), IDV(S), LPV(S), NFV(S), SQV(S), TPV(S) |
|                  |         | 3TC(S), ABC(S), AZT(S), D4T(S), DDI(S), FTC(S), TDF(S) |
|                  |         | DLV(S), EFV(S), ETV(S), NVP(S) |
| KRC5180          | B       | None/ None/ None/ HAART naive/ primary patient |
|                  |         | ATV(S), DRV(S), FPV(S), IDV(S), LPV(S), NFV(S), SQV(S), TPV(S) |
|                  |         | 3TC(S), ABC(S), AZT(S), D4T(S), DDI(S), FTC(S), TDF(S) |
|                  |         | DLV(S), EFV(S), ETV(S), NVP(S) |
| KRC5123          | B       | None/ None/ None/ HAART naive |
|                  |         | ATV(S), DRV(S), FPV(S), IDV(S), LPV(S), NFV(S), SQV(S), TPV(S) |
|                  |         | 3TC(S), ABC(S), AZT(S), D4T(S), DDI(S), FTC(S), TDF(S) |
|                  |         | DLV(S), EFV(S), ETV(S), NVP(S) |
| KRC1097          | B       | None/ D67N, K70R, T215I, K219Q, K103R/ |
|                  |         | ATV(S), DRV(S), FPV(S), IDV(S), LPV(S), NFV(S), SQV(S), TPV(S) |
|                  |         | 3TC(S), ABC(L), AZT(H), D4T(I), DDI(L), FTC(S), TDF(L) |
|                  |         | DLV(S), EFV(S), ETV(S), NVP(S) |
| KRB9149          | B       | None/ HAART experienced |
|                  |         | ATV(H), DRV(L), FPV(H), IDV(H), LPV(H), NFV(H), SQV(H), TPV(I) |
|                  |         | 3TC(H), ABC(H), AZT(H), D4T(H), DDI(H), FTC(H), TDF(I) |
|                  |         | DLV(S), EFV(S), ETV(S), NVP(S) |
| KRB7021          | B       | None/ HAART experienced |
|                  |         | ATV(I), DRV(L), FPV(H), IDV(H), LPV(H), NFV(H), SQV(I), TPV(L) |
|                  |         | 3TC(H), ABC(H), AZT(H), D4T(H), DDI(H), FTC(H), TDF(I) |
|                  |         | DLV(I), EFV(H), ETV(S), NVP(H) |

\(^a\)Drug resistance was estimated using the Stanford University Genotypic Resistance Algorithm (HIVdb).

HAART = highly active antiretroviral therapy; NRTI = nucleoside reverse transcriptase inhibitor; NNRTI = non-nucleoside reverse transcriptase inhibitor; PI = protease inhibitor; H = High-level resistance; I = Intermediate resistance; L = Low-level resistance; S = Susceptible.
cells were then cultured at 37°C in 5% CO₂ and 48 hours after infection, β-galactosidase activity was measured using X-gal staining.

3. Results

3.1. Genetic characteristics of the resistance gene from clinical HIV-1 isolates

The HIV-1 isolates that were obtained from two HAART-naïve HIV/AIDS patients (KRC5180, KRC5123) and three HAART-experienced patients (KRC1097, KRB9149, KRB7021) out of the pool of samples sent from hospitals to Korea National Institute of Health in 2006 requesting a genotypic assay of antiretroviral drug-resistant variants are listed in the Table. All of five clinical isolates of the HIV-1 strains were of the HIV-1 subtype B.

Based on the genotypic drug resistance assay, none of the isolates from the HAART-naïve patients (KRC5180, KRC5123) were resistant to the antiretroviral drugs. KRC5180, a patient with a primary infection, had isolates that were protease inhibitors (PIs)-related minor resistant variants like L10I and L63P. The isolates from a HAART-experienced patient (KRC1097) had antiretroviral drug-resistant sites, similar to those for the AZT-resistant reference strain (RTMC), at D67N, K70R, T215I, and K219Q in the protein coded by the thymidine-related drug-resistant gene. The isolates from KRB9149 and KRB7021 were found to have antiretroviral drug resistance-related polymorphic sites related to reverse transcriptase inhibitors (RTIs) resistance at M41L, D67N, L74I, V75T/M, V118I, M184V, L210W, T215Y, K219N, Y188L, and K238Q as well as sites related to PIs-related resistance at M46I, G48V, I50V, I54A, V82T, V32I, M46I, I47A, and L90M (see the Table for details).

3.2. Levels of p24 antigen in the homologous recombinant HIV-1 pseudo-virus propagated in the C8166 cell line

The levels of the p24 antigen of homologous recombinant HIV-1 pseudo-viruses propagated using C8166 cell lines compared with the level in the reference strain HXB2 are shown in Figure 1. Four of the five isolates showed p24 levels following as; 65.56 pg/mL, 62.64 pg/mL, 55.64 pg/mL, and 52.37 pg/mL for KRC5180, KRB9149, KRB7021, and KRC5123, respectively. In comparison with reference strain HXB2 (61.87pg/mL), clinical isolates were in the similar range (61.87 pg/mL ± 9.5pg/mL). However, the pseudo-virus derived from KRC1097 showed a very low level of p24 antigen production (8.02 pg/mL) (Figure 1). The HIV-1 homologous recombinant virus has the RTI resistance-related sites, D67N, K70R, T215I, K219Q and K103R.

4. Discussion

Many different methods have been used to measure virus replication capacity [10,11]. Cell-based phenotypic
assays help to determine the effect of viral variants on antiretroviral drug resistance [12,13]. However, phenotypic assays are limited to measuring mixed HIV-1 variants with high resistance measurements and their use in finding the resistance-related site is limited [14]. In this study, the replication capacity of the HIV-1 recombinant virus derived from patients was measured using homologous recombination [8]. The viral replication capacity has been measured using the expression levels of the HIV-1 p24 antigen [15]. However, because of the limitations of the phenotypic assay, measuring the replication capacity using p24 antigen levels is restricted to match the virus’s replication mechanism in the patient.

In the present study, the replication capacity or infectivity of HIV-1 drug-resistant variants derived from HAART-experienced patients was investigated to understand the characteristics of drug-resistant strains in Korea. Therefore, to estimate replication capacity, p24 antigen levels were measured in recombinant cell lines using clinical isolates from HAART-experienced patients, HAART-naïve patients and the reference strain HXB2. The HIV-1 p24 antigen level was the lowest in KRC1097, a HAART-experienced patient with antiretroviral drug resistance-related polymorphic sites at D67N, K70R, T215I, K219Q, and K103R in the protein coded by the thymidine-related drug-resistant gene. KRB7021 with M41L, V118I, M184V, L210W, T215Y, K219N, Y188L, and K238Q mutations related to RTIs resistance had the next lowest p24 antigen level. On the other hand, the pseudo-virus derived from a HAART-naïve patient showed a high level of the p24 antigen compared with the reference strain HXB2. However, low p24 antigen levels were not seen only in the drug-resistant variants; isolates from the HAART-naïve patient KRC5123, also had low p24 antigen levels as did isolates from KR89149.

We also measured pseudo-virus infectivity using TZM-bl cell lines. Virus infectivity was lower in drug-resistant variants than in the reference strain HXB2. The infectivities of homologous recombinant pseudo-viruses were highest in the reference strain HXB2 followed by the isolates from HAART-naïve patients (KRC5180 > KRC5123), and finally by the isolates from the HAART-experienced patients (KRB7021 > KR89149 > KRC1097). In general, the antiretroviral drug-resistant variants in this study showed lower infectivity compared with HXB2 or isolates derived from HAART-naïve patients. This result showed that the drug-resistant HIV-1 variants had lower infectivity and suggested that their replication capacity in the host may be low compared with the wild type or with the reference strain HXB2. The isolate from KRC1097 had a T215I mutation. Mutations at codon 215 (T215A/C/D/E/G/H/I/L/N/S/V) are known to increase the risk of virologic failure of AZT or stavudine in HAART-naïve patients. The T215Y mutant may emerge quickly from one of these mutations in the presence of AZT or stavudine [16]. Furthermore, a decrement in replication capacity influenced by drug resistance mutations has been reported [17]. Therefore, the low infectivity of isolates derived from KRC1097 may be related to the revertant mutation from T215Y to T215I as well as the thymidine analog-associated mutations such as M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E [16]. More investigations using clinical isolates are needed to more fully understand this effect.

In a drug-resistant variants surveillance report in Korea, multi-nucleoside reverse transcriptase inhibitor-resistant variants were frequently described with mutation sites of M41L, D67N, K70R, L210W, T219Q/E in HAART-experienced patients [6,16] and besides M41L, D67N, and K70R, other mutation sites, L74V, M184V, V118I, L100I and K103N, were found in HAART-experienced HIV/AIDS patients [6]. It has also been reported that the interaction of drug-resistant variants affect HIV-1 replication capacity [1]; however, this needs to be further investigated using more clinical isolates.

For clinical isolates with no drug-resistant variants, the variants that do exist are susceptible to antiretroviral drugs, keeping the viral load in the blood at a low level. However, drug-resistant variants have low infectivity compared with the reference strain and the viral load in the blood is expected to be high. Thus, it is supposed that antiretroviral drug as booster for viral load or else. Drug resistance is currently analyzed in clinical isolates by genotypic assay based on drug treatment trends. Thus, the present study may contribute to understanding the viral characteristics of RT mutation strains at the cellular level. Recently, the single cycle assay has been used with a replication-deficient recombinant virus containing the indicator gene (luciferase, β-galactosidase) [5,13,18]. By generating site-specific mutations, single cycle assays are expected to be used to elucidate the effects of site-specific mutations on the viral mechanism [14,18].

In this study, we examined the infectivity of patient-derived pseudo-viruses in the absence of antiretroviral drugs. For further study, we need to investigate the differences between viral infectivity and drug susceptibility in the presence of antiretroviral drugs.

Furthermore, the relationship between viral replication capacity and the RT and PR clinical mutation variants under drug treatment condition needs to be investigated. Such an investigation would contribute to our understanding of the characteristics of drug-resistant variants that arise in response to drug treatment.

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