A simple and cost-saving phenotypic drug susceptibility testing of HIV-1

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It is essential to monitor the occurrence of drug-resistant strains and to provide guidance for clinically adapted antiviral treatment of HIV/AIDS. In this study, an individual patient’s HIV-1 pol gene encoding the full length of protease and part of the reverse transcriptase was packaged into a modified lentivirus carrying dual-reporters ZsGreen and luciferase. The optimal coefficient of correlation between drug concentration and luciferase activity was optimized. A clear-cut dose-dependent relationship between lentivirus production and luciferase activity was found in the phenotypic testing system. Fold changes (FC) to a wild-type control HIV-1 strain ratios were determined reflecting the phenotypic susceptibility of treatment-exposed patient’s HIV-1 strains to 12 HIV-1 inhibitors including 6 nucleoside reverse-transcriptase inhibitors (NRTIs), 4 non-nucleoside reverse transcriptase inhibitors (NNRTIs) and 2 protease inhibitors (PIs). Phenotypic susceptibility calls from 8 HIV-1 infected patients were consistent with 80–90% genotypic evaluations, while phenotypic assessments rectified 10–20% genotypic resistance calls. By a half of replacement with ZsGreen reporter, the consumption of high cost Bright-Glo Luciferase Assay is reduced, making this assay cheaper when a large number of HIV-1 infected individuals are tested. The study provides a useful tool for interpreting meaningful genotypic mutations and guiding tailored antiviral treatment of HIV/AIDS in clinical practice.

During the past decades, the numbers of HIV-1 inhibitors have been approved for the treatment of HIV-1 infected patients. The highly active antiretroviral therapy (HAART), which targeted the reverse transcriptase (RT) and/or protease of HIV-1 by combination of two or more inhibitors, was considered one of the most cost-effective therapeutic interventions for HIV-1 infected patients1. It had been shown not only to improve the quality of life of HIV-1 infected patients but also to reduce the risk of HIV-1 dissemination2,3. Despite their capacity of suppressing viral production and reducing the rate of virus transmission, HAART failed to eradicate the virus from HIV-1 infected patients4. Consequently, the clinical benefits of antiretroviral therapy had been compromised by the emergence of HIV-1 drug-resistant strains primarily due to escape virus variants under the drug selection pressure5. Therefore, it is essential to monitor the occurrence of drug-resistant strains and to provide guidance for clinically adapted antiviral treatment.

Currently, there are two types of genotypic and phenotypic drug resistance tests available for monitoring the HIV-1 drug resistance. The genotypic resistance test that can detect specific resistance-related mutations in the target genes of HIV-1 by DNA sequencing, is more frequently used than the phenotypic test due to ease to perform, low costs and interpretation algorithms available online6,7. However, the genotypic assay can be difficult to interpret resistance levels when it comes to sequences with unusual mutations or complex patterns of mutation combinations8. The phenotypic resistance test, which can detect the viral production or the enzymatic activity affected by HIV-1 inhibitors in vitro, has the ability to directly measure the drug resistance level or susceptibility of each inhibitor without any prior knowledge of mutations in HIV-1 strains. Therefore, it is considered significant that phenotypic susceptibility testing provides a precise guidance of drug usage for HIV-1 infected patients, especially patients exposed to antiviral drugs9. Usually, the phenotypic susceptibility assays are performed by

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culture of clinical strains. However, these assays require fresh peripheral blood mononuclear cells (PBMCs) from donors, are labor-intensive and time-consuming. Recently, several assays either based on a recombinant virus with single-loop infection or infectious particles with multiple cycles of replication were developed. Although those assays show great potential in phenotypic drug susceptibility testing, their cost remains high due to the expensive Bright-Glo Luciferase Assay System and concerns regarding bio-safety.

In this study, we developed a novel lentivirus-based phenotypic assay with two reporters: luciferase and ZsGreen. The main advantage of dual reporters is that the viral response to HIV-1 inhibitors is easily observed either by cells stained with ZsGreen under the Inverted Fluorescence Microscope or by quantification of luciferase activity. In addition, the HIV-1 based lentivirus being known powerful and safe is an ideal vector for HIV-1 drug resistance testing. Using this new assay, the phenotypic susceptibility level of HIV-1 in infected patients was measured and analyzed against a panel of antiviral drugs available in China.

**Results**

**A novel recombinant lentivirus system presenting phenotypic drug susceptibility of HIV-1.**

The modified lentivirus packaging system consisted of three plasmids with envelope, packaging and transfer functions carrying a patient’s HIV-1 pol gene and dual reporter genes of luciferase and ZsGreen (Supplemental Figs S1 and S2). The patient’s HIV-1 pol gene within packaging plasmid psPAX2m-Pol was integrated into recombinant lentiviral genome with envelop plasmid pMD2.G and transfer plasmid pHAGE-CMV-Luc-IRES-ZsGreen in 293FT cells (Fig. 1). The patient’s HIV-1 derived lentivirus could functionally present the drug susceptibility by luciferase or ZsGreen reporters in the transduced or infected 293A cells in the presence of different concentrations of antiviral drugs.

**Establishment of optimized MOI, cell density and time point in phenotypic drug susceptibility test.** In order to establish optimal assay conditions, the multiplicity of infection (MOI), cell density and time point of detection were optimized with a representative HIV-1 inhibitor in phenotypic susceptibility testing. First, a titer of patient’s HIV-1 derived lentivirus was determined within a range of virus dilution folds around 10% ZsGreen positive rate from lentivirus-transduced 293A cells. Secondly, the lentivirus with ZsGreen reporter was used for selecting suitable drug dilution range and pre-titration of the MOI (Fig. 2), in which the drug dilutions corresponding to 10–90% ZsGreen positive cells were primarily chosen for further titration of MOI by the lentivirus with luciferase reporter. When the concentration of Zidovudine (a representative of NRTIs and NNRTIs) decreased in the presence of lentivirus transduced 293A cells, the positive rate of ZsGreen cells increased (Fig. 2A), in which the drug destroyed the function of HIV-1 pol and inhibited the expression of ZsGreen in the virus infection process. In a different mechanism from NRTIs or NNRTIs, when the concentration of Lopinavir (a representative of PIs) decreased in the presence of lentivirus transduced 293A cells, the positive rate of ZsGreen cells increased since Lopinavir prevents maturation of the internal structural proteins and of the viral enzymes in a dose-dependent manner.

The luciferase activity increased from 2 to 20 MOI (Fig. 3A), the optimal coefficient of correlation (R² = 0.958, P < 0.01) between drug concentration and luciferase activity being 10 MOI. Meanwhile, between five different cell densities tested (1 × 10⁴, 1.5 × 10⁴, 2 × 10⁴, 2.5 × 10⁴ and 3 × 10⁴ cells/well), the highest R²(0.998, P < 0.01) was observed with 1.5 × 10⁴ cells/well (Fig. 3B). In addition, the optimal detection time was 60 h after lentivirus inoculation since not only it gave the highest R²(0.938, P < 0.01) between drug concentration and luciferase activity.
but also the second highest activity of luciferase (Fig. 3C). Finally, stability and reproducibility of the phenotypic resistance testing were evaluated by determining the concentration of 12 antiviral drugs at 50% inhibition (IC50) of lentivirus infection in quadruplicate from three separate tests (Table 1). The data appeared highly reproducibility and stability with coefficient of variation (CV) below 15%.

Analysis of genotypic drug resistance-associated mutations from patient’s HIV-1 strains. The HIV-1 infected patient information was indicated in Table 2, showing that all patients were previously treated with a combination of antiviral drugs. All eight patients had CD4 cell counts below 400 cells/mm³ and five of them below 200 cells/mm³. Patient’s HIV-1 RNA load ranged between 155 and 104,000 copies/ml. To analyze the drug resistance associated with viral mutations from these patients, the HIV-1 pol genes were amplified and sequenced. Based on the genotypic resistance database (the Stanford University HIV Drug Resistance Database) available for 10 HIV-1 inhibitors involved in this study, the mutations from those HIV-1 strains were associated with genotypic drug resistance to NRTIs, NNRTIs and PIs, respectively (Table 3). The antagonistic mutations K65R and M184V were found from two patients ID 38290075 and 38290022, respectively (Table 3).
K65R from the patient ID 38290075 was previously reported susceptible to zidovudine (AZT) and stavudine (D4T), but decreased the susceptibility of HIV-1 to tenofovir, didanosine, abacavir and lamivudine. Mutation M184V from the patient ID 38290022 was described partly susceptible to tamoxifen (TAM), zidovudine, stavudine and tenofovir, but increased the sensitivity of HIV-1 to these antiviral agents. When however, it comes to analyze the drug resistance of HIV-1 strains which carried such mutations, they might be predicated resistant by genotypic resistance testing, while the phenotype of drug resistance were actually differed. Therefore, without massive correlative information of genotypic and phenotypic resistance testing of various viral mutants to individual drugs, the genotype testing was not sufficient to predict the drug resistance levels from the viral replication effect of complex mutations as well as new mutations of HIV-1 strains.

Detection of phenotypic drug susceptibility from patients’ HIV-1 strains.

Eight HIV-1 strains were measured for drug susceptibility using the recombinant lentivirus system with dual reporters (Supplemental Fig. S3). A wildtype lentivirus whose gag and pol genes are derived from the subtype B strain served as control for calculation of the fold changes (FC). The phenotypic drug susceptibility or resistance level to 12 antiviral drugs are shown in Table 1. Comparing with genotypic resistance analysis available for 10 drugs from the Stanford University HIV Drug Resistance Database (http://hivdb.stanford.edu/), the majority of phenotypic testing calls were concordant with genotypic assessments (Table 1). Approximately 21% (17/80) discrepancy rate was observed between phenotypic and genotypic drug resistance testing from these HIV-1 strains, of which 10% (8/80) drug resistance calls clearly differed by nearly two levels among susceptibility (S), low (L), middle (M) and high (H) resistance levels between two assays (Table 1). For instance, the patient’s HIV-1 strain 38290022 was susceptibility to DDI and D4T drugs and high resistance to RPV by phenotype, but middle or high resistance to DDI and D4T and low resistance to RPV by genotype, while HIV-1 strain 38290312 was highly resistant to ETR by phenotype but low resistance by genotype. The results suggested that the newly developed phenotypic susceptibility testing could rectify at least 10% of incorrect calls regarding drug resistance levels predicted by genotypic testing.

Table 1. Reproducibility of phenotypic resistance test for 12 antiviral drugs. IC50, the drug concentration producing 50% inhibition of positive ZsGreen cells; SD, standard deviation; CV, coefficient of variation.

*The concentration of these drugs is presented in nmol/L. The data were obtained from three representative experiments in quadruplicate.

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Discussion

Drug resistance level or susceptibility testing is considered an important issue for the management of newly HIV-1 infected and treatment-exposed HIV-1 infected patients. When a patient was newly diagnosed with
HIV-1 treatment failure, drug susceptibility testing is recommended by the US Department of Health and Human Services (DHHS), International AIDS Society (IAS-USA) and European guidelines21. Phenotypic susceptibility assay is optimal to provide accurate and quantitative assessments of HIV-1 strains susceptible to a large number of antiviral drugs, especially for patients receiving antiretroviral for years9,22,23. The principle of phenotype testing is to quantify in vitro viral replication in the presence of serial dilutions of antiviral drugs. Drug resistance level (susceptibility) is estimated as FC calculated from a ratio of the IC50 of the patient strain to the IC50 of a wild-type control virus.

Several phenotypic assays have been previously described for assessing HIV-1 drug resistance in clinical conditions, including the first-generation Antivirogram and PhenoSense assays14,24,25, the ExaVir™ Drug assay26,27, the modified assays with a single cycle system or single-loop infection11–13, with two round infection28 or multiple cycles of replication13. In our study, a second-generation lentivirus vector carrying individual patient’s HIV-1 pol gene was constructed, in which the phenotypic drug-resistance was represented by dual-reporters in a single lentiviral vector with single restriction sites of Apa I and Age I appeared to be universally capable of integrating a drug-targeting pol gene from individual HIV-1 strains. The phenotypic drug-resistance was confirmed by the ExaVir™ Drug assay26,27, the modified assays with a single cycle system or single-loop infection11–13, with two round infection28 or multiple cycles of replication13. In this study, phenotypic susceptibility testing results were consistent with 80–90% genotypic interpretations obtained from the Stanford University HIV drug resistance database28, as reported in previous studies13,30. However, approximately 10–20% discrepant resistance calls from 10 drugs were found between the two assays31,32. Those discrepancies might be related to three major reasons. Firstly, many drug resistance mutations obtained by sequencing arise in complex patterns including the most common variants, which make it difficult for interpreting the correlation with HIV-1 susceptibility phenotype33. For example, mutation M41L is a thymidine analog mutation (TAM) that usually occurs together with T215Y and confers high-level resistance to D4T and AZT and middle-level resistance to DDI and ABC, respectively34. In our study, however, M41L was not accompanied with T215Y but with T215F in patient ID 38290022 (Table 4), while the patient ID 3829012 T215F was accompanied with M41L but not with T215Y in patient ID 38290312 (Table 4).

### Table 3. Analysis of drug resistance-associated mutations in HIV-1 strains from 8 patients

| Patient ID | NRTI resistance mutations | NNRTI resistance mutations | Other RT mutations | PI major resistance mutations | PI minor resistance mutations | Other PI mutations |
|------------|---------------------------|-----------------------------|--------------------|-------------------------------|-------------------------------|-------------------|
| 38290022   | M41L, M184V, T215F        | K101H, V106A, G190A, F227L  | K20R, V60I, A98S, D123E, A132V, H115Y, V179I, G196E, T200A, G207Q, K211V, K233Q, L228R, A272P, T275R, V756I | D60E, I62V, I65P, I77V, V77I, Y93L |                               |                   |
| 38290063   | D67N, T69D, K70R, M184V, T215F, K219Q | K103N, G190A                | K101Q, K122E, D123N, D177N, I178M, T200A, G207Q, K211K, E248D | A71T                          | N37T, R212, E618, I62V, L65P, I69K |
| 38290075   | K65R, K70R, V75I, F77L, Y115F, F116Y, Q151M, K219E | K103N, Y118C                | S68G, T69I, A172I, N176I, T200I, K211K, L228R, V230F, A272P, E297K, L318N |                               | I5V, K118, D60E, L63P |
| 38290079   | K103N                     | E6G, K11T, V35T, T39K, K43E, S68G, K122E, D123N, K166R, K173I, Q174E, D177E, I178M | L101F                          |                               | I5V, K20R, E535D, M361, M384, R41K, I62V, L65P, H69K |
| 38290243   | V90L, K101E, E138K        | E6P, K11R, K122E, A225T, V226I, G239K, A272P, V295I, H322L, E327K |                               |                               | T45, L15, N37Y, R41K, D60E, L63P |
| 38290306   | K103N, Y118C, P225H       | K11R, K102R, D123E, T200I, R211K, V245E, A272P, T296S, E297K | I5V, M36, N37Y, P39Q, R41K, K45R, D60E, L63P |                               |                   |
| 38290309   | E6G, V35I, G69K, K102S, I135V, I178G, G196E, T200E, E204K, L205Q, Q207E, A272P, I293V, E297K | A71V                          |                               |                               |                   |
| 38290312   | T215S                     | E6G, I135T, Q196E, R211K, D244V, G262E, A272S, K277R, A288S, I293V, E297K |                              | L90M                          | L101, A71T         | I13Y, K20R, E535D, M361, L64V, V77I, Y93L |
phenotype of this virus appeared susceptible to DDI and D4T (FC < 3) and remained moderately resistant to ABC (FC = 6-< 10), suggesting that phenotypic testing was more suitable to evaluate drug susceptibility than genotypic testing of resistance. Secondly, presence of antagonistic mutations may explain the discrepancy of resistance between genotypic and phenotypic assays. For example, M184V mutation is reported partly to reverse resistance to TAM, AZT, D4T and TDF, and increases susceptibility of HIV-1 to these inhibitors. In addition, revertant mutations T215S/C/D/E/I/V which usually do not reduce the resistance between genotypic and phenotypic assays. For example, M184V mutation is reported partly to reverse resistance to TAM, AZT, D4T and TDF, and increases susceptibility of HIV-1 to these inhibitors12. Phenotypic drug resistance is presented as the fold change (FC), of which FC = 3-< 6 low resistance, FC = 6-< 10 middle resistance, FC ≥ 10 high resistance of drug to HIV-1 strains, respectively.31,36.

### Materials and Methods

#### Patient samples and antiviral drugs.
Plasma samples from HIV-1 infected patients were provided by the center of disease prevention and control (CDC), Shenzhen, China. All patients had been treated with antiviral drugs in the past years. All participating patients signed an informed consent for sample collection and testing. This study was approved by the Medical Ethics Committee of Southern Medical University (permit numbers: NFYY-2008-045), Guangzhou, China. All experiments were carried out in accordance with the approved guidelines.

| HIV-1 strain | Type | DDI | D4T | AZT | DDC | FTC | ABC | NVP | ETR | DPV | RPV | NFV | LPV |
|-------------|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 38290022    | Phenotypic | 1.22 | 1.6 | 17.7 | 1.38 | >1000 | 6.7 | >1000 | 5 | 7.85 | 12.2 | 0.34 | 0.9 |
|             | Genotypic | M↑↑ | H↑↑ | H | NA | H | M | H | L | NA | L | M↑ | S |
| 38290063    | Phenotypic | 2.38 | 2 | 14.3 | 2.09 | >1000 | 3.95 | >1000 | 3.6 | 3.86 | 7.2 | 0.26 | 1.17 |
|             | Genotypic | H↑↑ | H↑↑ | H | NA | H | H↑↑ | H | L | NA | L | H | S |
| 38290075    | Phenotypic | >1000 | 51 | >1000 | 74 | >1000 | 97 | >1000 | 645 | >1000 | 215 | 2.98 | 1.57 |
|             | Genotypic | H | H | H | NA | H | H | H | H↑↑ | NA | M↑ | S |
| 38290079    | Phenotypic | 1.2 | 0.74 | 0.48 | 0.87 | 0.35 | 0.94 | 28 | 1.6 | 1.69 | 1.2 | 0.29 | 0.93 |
|             | Genotypic | S | S | S | NA | S | S | H | S | NA | S | S |
| 38290243    | Phenotypic | 1.1 | 1 | 0.4 | 1.75 | 1.85 | 0.8 | 4.23 | 15 | 8.83 | 7.32 | 1.04 | 2.46 |
|             | Genotypic | S | S | S | NA | S | S | S | S | M↑ | L↑ | NA | H↑ |
| 38290306    | Phenotypic | 0.92 | 0.86 | 0.3 | 0.7 | 0.66 | 0.8 | >1000 | 87 | 270 | 30.74 | 2.25 | 2.1 |
|             | Genotypic | S | S | S | NA | S | S | S | H | M↑ | NA | M↑ | S |
| 38290309    | Phenotypic | 1.07 | 1 | 1.4 | 1.07 | 0.97 | 0.74 | 0.5 | 2.7 | 1.37 | 2.08 | 1.35 | 2 |
|             | Genotypic | S | S | S | NA | S | S | S | S | S | S | S |
| 38290312    | Phenotypic | 1.02 | 0.68 | 0.95 | 0.6 | 0.89 | 1.15 | >1000 | 17.8 | 48.4 | 16.29 | 10 | 0.42 |
|             | Genotypic | S | L↑ | L↑ | NA | S | S | H | L↑ | NA | H | H | L↑ |
| Wild type   | Phenotypic | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
|             | Genotypic | S | S | S | NA | S | S | S | S | NA | S | S | S |

Table 4. Comparison of phenotypic and genotypic drug resistance testing of HIV-1 strains. NA, not available. Genotypic drug resistances are indicated by S (susceptible), L (low resistance), M (middle resistance) or H (high resistance) according to the database [Available at: http://hivdb.stanford.edu/; accessed on April 28, 2014 and February 20, 2016], of which capital letters in bold with "+" or "↓" and underline indicate discrepancies between phenotypic and genotypic resistance for at least one or two levels, respectively.
Twelve HIV-1 inhibitors were used in this study, including six nucleoside reverse-transcriptase inhibitors (NRTIs): Didanosine (DDI), Stavudine (D4T), Zidovudine (AZT), Zalcitabine (DDC), Emtricitabine (FTC), Abacavir Sulfate (ABC), four non-nucleoside reverse transcriptase inhibitors (NNRTIs): Nevirapine (NVP), Etravirine (ETR), Dapivirine (DPV), Rilpivirine (RPV), and two protease inhibitors (PIs): Nelfinavir (NFV) and Lopinavir (LPV), which were purchased commercially from Selleckchem (Shanghai, China).

Isolation of drug-targeting genes of HIV-1. Viral RNA was extracted from HIV-1 infected patient's plasma samples with High Pure Viral Nucleic Acid Kit according to the manufacturer's instructions (Roche, Pleasanton, USA). The HIV-1 pol genes encoding protease 1-99 amino acids and reverse transcriptase 1-314 amino acids were amplified by RT nested-PCR using PrimeScript™ II High Fidelity RT-PCR Kit (TaKaRa, Dalian, China). The primer sets were as follows13: Outer-F, 5′-GCAAGAGTTTTTGCTGAAGCAATGAG-3′; Outer-R, 5′-CCTTGCCCTCCTGCTTATTTTGC-3′; Inner-F, 5′-TGCAAGGGCCCTAAGGAAAAAGGGCTG-3′ (Apa I); Inner-R, 5′-CACTCCATGTACCCGTTCTTTTGAATCTC-3′ (Age I). The inner primers had added restriction sites Apal and AgeI, respectively. The first-round PCR was done at 45 °C for 30 min and 94 °C for 2 min followed by 30 cycles of 98 °C for 10 s, 55 °C for 15 s and 68 °C for 2 min. The second-round PCR was performed at 98 °C for 5 min followed by 30 cycles of 98 °C for 10 s, 51 °C for 15 s and 68 °C for 2 min. The patient-derived PCR products were purified by an AxyPrep DNA Gel Extraction Kit (AXYGEN, Suzhou, China) and sequenced by a commercial company (Invitrogen, Guangzhou, China). The sequenced PCR fragments were digested with Apal and AgeI, and were ready for cloning and expressing in the recombinant lentiviral vector system.

Recombinant lentiviral vector system with three plasmids carrying dual reporter genes. Packaging plasmid psPAX2m-Pol carried the patient HIV-1-derived pol gene. Plasmid psPAX2 (#12260) was a gift from Dr Didier Trono (Lausanne, Switzerland), which contained the pol genes from a wild-type HIV-1 subtype B NL4.3 strain (GenBank access number AF324493.2)10. In order to insert the drug resistance gene fragment (pol) of HIV-1 strains into the packaging plasmid psPAX2 at restriction sites Age I and Apa I, the vector was genetically modified for dysfunction of two extra sites of Age I (position nt 7697) and Apa I (position nt 863) by amplification and site-directed mutagenesis (Supplemental Table S1), designated as psPAX2m. The targeted drug resistance genes (which corresponds to the consensus sequences) from HIV-1 infected patients were individually cloned into the psPAX2m at Age I and Apa I restriction sites, which were designated as psPAX2m-A and were used for recombinant lentivirus production (Supplemental Fig. S1).

Envelop plasmid pMD2.G. The vector (plasmid #12259) was a gift from Didier Trono (Lausanne, Switzerland). Transfer plasmid pHAGE-CMV-Luc-IRES-ZsGreen contained dual-reporter genes. The CMV promoter appeared to initiate higher transduction efficiency than EF1α promoter in 293A cells34. In order to get a stronger signal of luciferase or ZsGreen, the EF1α promoter in the pHAGE-EF1α-IRES-ZsGreen (a gift from Jen-Shin Lee, Harvard Medical School, USA) was replaced by the CMV promoter pMD2.G. Briefly luciferase gene fragment from pGL3 Luciferase Reporter Vector (Promega, Beijing, China) was cloned into the pHAGE-CMV-IRES-ZsGreen next to the IRES. The transfer vector with dual-reporters was designated pHAGE-CMV-Luc-IRES-ZsGreen and suitable for lentivirus production (Supplemental Fig. S2).

Recombinant lentivirus production and titration. A total of 3 × 10⁵ 293FT cells were plated in 75 cm² cell culture flask 36 h prior to transfection. When 75% cell confluence was reached, cells were co-transfected with a total of 15μg of three plasmid DNAs (2.8μg envelop plasmid pMD2.G, 5.2μg packaging plasmid psPAX2m-Pol and 7μg transfer plasmid pHAGE-CMV-Luc-IRES-ZsGreen) and 45μl of X-tremeGENE HP DNA Transfection Reagent (Roche, USA) diluted in 1.5 ml of Opti-MEM medium and incubated at room temperature for 15 min. After cell culturing for 48 h at 37 °C, the lentivirus-containing supernatant was harvested after centrifugation at 3000 rpm at 4 °C for 20 min and filtration with a Millex-HV 0.45 μm filter (Millipore, USA). Lentivirus titration was performed as a previously described assay with modifications39. Briefly, a total of 5 × 10⁶ 293A cells were seeded in 6-well plate (Corning, USA), and cultured for 24 h. The cells were inoculated in a series of lentivirus and incubated for 12 h. The cell medium was changed completely, replaced with fresh medium, and further incubated for 48 h. Cells in each well were counted by flow cytometry in order to enumerate the rate of ZsGreen positive cells. The titer was determined by the following formula: (F × C/V) × D. F = frequency of ZsGreen-positive cells (the percentage obtained and divided by 100), C = total number of cells in the well at the time of infection, V = volume of lentivirus diluent in ml, D = lentivirus dilution fold. Following the formula and standardizing the results, lentivirus dilution fold was determined with a range of 1–30% GFP-positive cells observed after viral transduction.

Optimization of phenotypic drug susceptibility test. The lentivirus produced by co-transfection with psPAX2m, pMD2.G and pHAGE-CMV-Luc-IRES-ZsGreen served as a wild-type HIV-1 control of phenotypic resistance since the pol gene within psPAX2m was derived from a wild-type HIV-1 subtype B NL4.3 strain susceptible to HIV-1 inhibitors. To see whether the HIV-1 inhibitor affected the viral infection and protein production, Zidovudine (D4T) was used as a representative antiviral drug for optimizing the testing conditions according to a previous study12. The multiplicity of infection (MOI), cell density and time points were optimized to obtain the most suitable luciferase activity in the test. The luciferase activity was measured using the Bright-Glo Luciferase Assay System (Promega, USA) in a micro-well plate reader (Wallak 1420, Perkin Elmer, USA). Each data point was tested in quadruplicate. The susceptibility of HIV-1 pseudotyped lentivirus to those 12 antiviral drugs was determined at three different time points, and the reproducibility of testing was evaluated.
Phenotypic drug susceptibility testing for patient’s HIV-1 strains. The amplified HIV-1 pol gene sequences were submitted to the Stanford HIV Drug Resistance Database. The genotypic drug resistance of HIV-1 from the treated patients was analyzed with the HIVdb program available from the Stanford University HIV Drug Resistance Database. Based on the aforementioned results, phenotypic drug susceptibility testing for patient’s HIV-1 samples was performed using the newly developed lentivirus-based assay. A dose of 10^4 MOI of recombinant lentivirus with an individual targeting HIV-1 pol gene in the presence of different concentrations of NRTIs or NNRTIs were applied to infect 1.5 × 10^5 293A cells per well within 96 wells of cell culture plate in the test, and then the activity of luciferase was determined 60 h post-infection. All antiviral drug testing were performed in quadruplicate each test resulting from three representative experiments.

The antiviral effect of protease inhibitors (PIs) was measured during viral production. Briefly, 4 × 10^4 293FT cells/100 μl were seeded in each well of 96-well plate and incubated for 36 h. The cells were transfected with 15 μl of mixture with three plasmid DNAs in Opti-MEM and X-tremeGENE HP DNA Transfection Reagent (Roche, USA), and then 8 h later the cell culture was changed with 100 μl of fresh medium containing a 3-fold serial concentrations of PIs. After 48 h of incubation of the transfected cells with PIs, cells were spun down by centrifugation at 4000 rpm for 20 min at 4°C. Fifty μl of virus-containing supernatant were added to the 24h pre-seeded 293A cells for measuring the activity of luciferase after 60 h incubation from lentivirus transduced cells, respectively.

Data analysis. The percentage of inhibition was calculated using the following formula: Viral inhibition rate (%) = (1 – RLU in the drug treated group/RLU in the control) × 100% (RLU, relative luciferase activity unit). The drug concentration producing 50% inhibition (IC50) on an individual HIV-1 strain was calculated using nonlinear regression analysis. The phenotypic resistance level of a detected HIV-1 strain to an individual antiviral inhibitor was expressed as a fold-change (FC), the ratio of the IC50 of the tested strain to the IC50 of a wild-type strain control. The adjusted criteria for classifying the phenotypic resistance levels (susceptibility) of antiviral drugs to HIV-1 strains were defined as a ratio of FC < 3 indicative of drug susceptibility (S), FC = 3–< 6 indicative of low drug resistance (L), while FC = 6–< 10 and FC ≥ 10 indicative of intermediate or middle (M) and high drug resistance (H), respectively. All analyses were done with the software SPSS version 21.0.

References
1. Piacenti, F. J. An Update and Review of Antiretroviral Therapy. Pharmacotherapy. 26, 1111–1133 (2006).
2. Granich, R. et al. Highly Active Antiretroviral Treatment as Prevention of HIV Transmission: Review of Scientific Evidence and Update. Curr Opin HIV AIDS. 5, 298–304 (2010).
3. Burgoyne, R. W. & Tan, D. H. Prolongation and Quality of Life for HIV-infected Adults Treated with Highly Active Antiretroviral Therapy (HAART): A Balancing Act. J Antimicrob Chemother. 61, 469–473 (2008).
4. Pierson, T., McArthur, J. & Siliciano, R. F. Reservoirs for Viral Persistence in the Presence of Antiviral Immune Responses and Antiretroviral Therapy. Ann Rev Immunol. 18, 665–708 (2000).
5. Garcia-Lerma, J. G. & Heneine, W. Resistance of Human Immunodeficiency Virus Type 1 to Reverse Transcriptase and Protease Inhibitors: Genotypic and Phenotypic Testing. J Clin Virol. 21, 197–212 (2001).
6. Melikian, G. L. et al. Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI) Cross-Resistance: Implications for Preclinical Evaluation of Novel NNRTIs and Clinical Genotypic Resistance Testing. J Antimicrob Chemother. 69, 12–20 (2014).
7. Vercauteren, J. & Vandamme, A. M. Algorithms for the Interpretation of HIV-1 Genotypic Drug Resistance Information. Antiviral Res. 71, 333–342 (2006).
8. Paolucci, S. et al. Comparison of Levels of HIV-1 Resistance to Protease Inhibitors by Recombinant Versus Conventional Virus Phenotypic Assay and Two Genotypic Interpretation Procedures in Treatment-Naive and HAART-experienced HIV-infected Patients. J Antimicrob Chemother. 51, 135–139 (2003).
9. MacArthur, R. D. Understanding HIV Phenotypic Resistance Testing: Usefulness in Managing Treatment-Experienced Patients. AIDS Rev. 11, 223–230 (2009).
10. Japour, A. J. et al. Standardized Peripheral Blood Mononuclear Cell Culture Assay for Determination of Drug Susceptibilities of Clinical Human Immunodeficiency Virus Type 1 Isolates. The RV-43 Study Group, the AIDS Clinical Trials Group Virology Committee Resistance Working Group. Antimicrob Agents Chemother. 37, 1095–1101 (1993).
11. Petropoulos, C. J. et al. A Novel Phenotypic Drug Susceptibility Assay for Human Immunodeficiency Virus Type 1. Antimicrob Agents Chemother. 44, 920–928 (2000).
12. Wu, S., Yan, P., Yan, Y., Qu, L. & Xie, M. A Single-Loop Recombinant Pseudotyped-Virus-Based Assay to Detect HIV-1 Phenotypic Resistance. Arch Virol. 160, 1385–1395 (2015).
13. Jia, Z. et al. Phenotypic Analysis of HIV-1 Genotypic Drug-Resistant Isolates From China, Using a Single-Cycle System. Mol Diagn Ther. 15, 293–301 (2011).
14. Hertogs, K. et al. A Rapid Method for Simultaneous Detection of Phenotypic Resistance to Inhibitors of Protease and Reverse Transcriptase in Recombinant Human Immunodeficiency Virus Type 1 Isolates From Patients Treated with Antiretroviral Drugs. Antimicrob Agents Chemother. 42, 269–276 (1998).
15. Chang, S. et al. Comparison of Susceptibility of HIV-1 Variants to Antiretroviral Drugs by Genotypic and Recombinant Virus Phenotypic Analyses. Int J Infect Dis. 37, 86–92 (2015).
16. Zufferey, R. et al. Self-inactivating Lentivirus Vector for Safe and Efficient in vivo Gene Delivery. J Virol. 72, 9873–9880 (1998).
17. Upreti, D., Pathak, A. & Kung, S. K. Lentiviral Vector-Based Therapy in Head and Neck Cancer (Review). Oncol Lett. 7, 3–9 (2014).
18. McColl, D. J., Chappell, C., Parkin, N. T. & Miller, M. D. Prevalence, Genotypic Associations and Phenotypic Characterization of K65R, L74V and Other HIV-1 RT Resistance Mutations in a Commercial Database. Antivir Ther. 13, 189–197 (2008)
19. Ring, A. & Dowsett, M. Mechanisms of Tamoxifen Resistance. Endocr Relat Cancer. 11, 643–658 (2004).
20. Grant, P. M. & Zolopa, A. R. The Use of Resistance Testing in the Management of HIV-1-infected Patients. Curr Opin HIV AIDS. 4, 474–480 (2009).
21. Tang, M. W. & Shafer, R. W. HIV-1 Antiretroviral Resistance: Scientific Principles and Clinical Applications. Drugs. 72, e1–e25 (2012).
22. Fehr, J. et al. Replicative Phenotypic Addins Value to Genotypic Resistance Testing in Heavily Pre-Treated HIV-infected Individuals–The Swiss HIV Cohort Study. J Transl Med. 9, 14 (2011).
23. Doherty, W., Deville, J. G., Zilbermann, J. L. & Viani, R. M. HIV-1 Phenotypic Drug Resistance Testing Among Highly Treatment Experienced and Poorly Adherent Youth. Pediatr Infect Dis J. 32, 1158 (2013).
24. Zhang, J., Rhee, S. Y., Taylor, J. & Shafer, R. W. Comparison of the Precision and Sensitivity of the Antivirogram and PhenoSense HIV Drug Susceptibility Assays. J Acquir Immune Defic Syndr. 38, 439–444 (2005).
25. Pattery, T. et al. Development and Performance of Conventional HIV-1 Phenotyping (Antivirogram(R)) and Genotype-Based Calculated Phenotyping Assay (virco(R)TYPE HIV-1) On Protease and Reverse Transcriptase Genes to Evaluate Drug Resistance. *Inter virology*. 55, 138–146 (2012).

26. Napravnik, S., Cachafeiro, A., Stewart, P., Eron, J. J. & Fiscus, S. A. HIV-1 Viral Load and Phenotypic Antiretroviral Drug Resistance Assays Based On Reverse Transcriptase Activity in Comparison to Amplification Based HIV-1 RNA and Genotypic Assays. *J Clin Virol*. 47, 18–22 (2010).

27. Agneskog, E., Nowak, P., Kallander, C. F. & Sonnerborg, A. Evaluation of Etravirine Resistance in Clinical Samples by a Simple Phenotypic Test. *J Med Virol*. 85, 703–708 (2013).

28. Puertas, M. C. et al. Novel Two-Round Phenotypic Assay for Protease Inhibitor Susceptibility Testing of Recombinant and Primary HIV-1 Isolates. *J Clin Microbiol*. 50, 3999–3916 (2012).

29. Rhee, S. Y. et al. Human Immunodeficiency Virus Reverse Transcriptase and Protease Sequence Database. *Nucleic Acids Res*. 31, 298–303 (2003).

30. Dunne, A. L. et al. Comparison of Genotyping and Phenotyping Methods for Determining Susceptibility of HIV-1 to Antiretroviral Drugs. *AIDS*. 15, 1471–1475 (2001).

31. Yang, J., Geng, W., Zhang, M., Han, X. & Shang, H. Discordance Between Genotypic Resistance and Pseudovirus Phenotypic Resistance in AIDS Patients After Long-Term Antiretroviral Therapy and Virological Failure. *J Basic Microbiol*. 54, 1120–1125 (2014).

32. Bronze, M. et al. HIV-1 Phenotypic Reverse Transcriptase Inhibitor Drug Resistance Test Interpretation is Not Dependent On the Subtype of the Virus Backbone. *PLoS One*. 7, e34708 (2012).

33. Liu, T. F. & Shafer, R. W. Web Resources for HIV Type 1 Genotypic-Resistance Test Interpretation. *Clin Infect Dis*. 42, 1608–1618 (2006).

34. Cases-González, C. E., Franco, S., Martínez, M. A. & Menéndez-Arias, L. Mutational patterns associated with the 69 insertion complex in multi-drug-resistant HIV-1 reverse transcriptase that confer increased excision activity and high-level resistance to zidovudine. *J Mol Biol* 365, 298–309 (2007).

35. da Silveira, A. A., Cardoso, L. P., Franciscio, R. B. & de Araújo Stefani, M. M. HIV type 1 molecular epidemiology in pol and gp41 genes among naive patients from Mato Grosso do Sul State, central western Brazil. *AIDS Res Hum Retroviruses* 28, 304–307 (2012).

36. Hanna, G. J. & D’ Aquila, R. T. Clinical Use of Genotypic and Phenotypic Drug Resistance Testing to Monitor Antiretroviral Chemotherapy. *Clin Infect Dis*. 32, 774–782 (2001).

37. Naldini, L. et al. In vivo gene delivery and stable transduction of nondoiring cells by a lentiviral vector. *Science*. 272, 263–267 (1996).

38. Zhang, L. et al. Recombinant Interferon-Gamma Lentivirus Co-Infection Inhibits Adenovirus Replication. *Ex Vivo*. *PLoS One*. 7, e42455 (2012).

39. Ngaí, S. C., Rosli, R., Nordin, N., Veerakumarasivam, A. & Abdullah, S. Lentivirus Vector Driven by Polybiquitin C Promoter without Woodchuck Posttranscriptional Regulatory Element and Central Polypurine Tract Generates Low Level and Short-Lived Reporter Gene Expression. *Gene*. 498, 231–236 (2012).

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Conceived and designed the experiments: C.L., Y.W. and L.Z. Performed the experiments: Y.W., L.Z., J.H., J.Z., P.L. and S.B. Analyzed the data: C.L., Y.W. and J.-P.A. Contributed to reagents, materials and analysis tools: Z.Y. Conceived and designed the experiments: C.L., Y.W. and L.Z. Wrote the paper: C.L., Y.W. and J.-P.A. All authors reviewed the manuscript.

Additional Information

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