Original Article

Identification of HIV-1 Genotypic Resistance in Patients on First-line Antiretroviral Therapy Using Polymerase Chain Reaction and Sequencing

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Objective The aim of the study was to evaluate the characteristics of HIV drug-genotypic resistance among patients taking first-line ARV regimens using polymerase chain reaction and sequencing, and guide to design optimal ARV regimens for these patients.

Methods HIV reverse transcriptase-encoded gene was amplified with RT-PCR and amplified PCR products were aligned and comparatively analyzed with HIV resistance database to find drug-resistance mutations.

Results Twenty-eight PCR products were amplified and sequenced successfully in 30 serum samples of recruited HIV-infected patients with virologic failure. The resistance rate was 96%, mutations in NRT region were found in 26 patients (93%), while mutations in NNRT region were found in 27 patients (96%). M184V was the most common mutation (86%), K65R was selected in 14% of recruited individuals and TAMs occurred in 50% of patients, which resulted in resistance to NRTIs. Y181C and V179D were the most common mutations in NNRTIs and prevalence was 43% (12/28) and 36% (10/28), respectively, which resulted in cross-resistance to NNRTIs due to low-genetic barrier.

Conclusions Virologic failure may occur in long-term administration of first-line ARV regimens, and drug-resistance mutations can be found in these patients, which resulted in resistance to first-line ARV regimens. We emphasized that HIV viral load assay and resistance assay were important tools to guide healthcare workers to design an optimal second-line ARV regimens for HAART-experienced individuals with virologic failure.

Key words: HIV; HAART; Mutation; Resistance; PCR

With the advent of era of free highly active antiretroviral therapy (HAART), the morbidity and mortality have been dramatically reduced in HIV-infected population in China.^{1,2} HAART would fail due to drug side effects and toxicities, suboptimal pharmacokinetics, adverse drug-drug interaction with concomitant medications, and poor patients' adherence,³ so adequate clinical management is necessary to prevent virologic failure and improve treatment outcome. The level of HIV viral load during antiretroviral (ARV) therapy is the best predictor of subsequent virologic failure, and HIV drug-resistance genotypic testing is recommended as the preferred method to guide selection of ARV regimens.^{3,4}

In 2002, Chinese government initiated its National

Free Antiretroviral Treatment Program (NFATP) as a pilot project, and HAART treatment was rapidly scaled up and become available now.^{5,6} The standard first-line ARV regimens recommended by NFATP consisted of two nucleoside reverse transcriptase inhibitors (NRTIs) and one non-nucleoside reverse transcriptase inhibitor (NNRTI), which include zidovudine (AZT) or stavudine (d4T) plus lamivudine (3TC) as two NRTIs, plus nevirapine (NVP) or efavirenz (EFV) as NNRTI. The standard second-line ARV regimens consisted of tenofovir dipivoxil (TDF) plus lamivudine plus lopinavir/ritonavir (LPV/r). In 2012, Chinese ARV guideline was revised and stavudine was no longer recommended as preferred choice in first-line ARV regimens due to its side effects, and was replaced by tenofovir dipivoxil. With increasing number of HAART treatment in Chinese HIV-infected patients, drug-resistant HIV strains have emerged and virologic failure has developed progressively.⁷

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The aim of this study was to evaluate the characteristics of HIV drug-genotypic resistance among patients taking first-line ARV regimens using polymerase chain reaction and sequencing, and guide to design optimal ARV regimens for these patients.

METHODS

The study received the approval of the Ethics Committee from Beijing Ditan Hospital, Capital Medical University, Beijing, China. All of the participants meeting the inclusion criteria had signed informed consent.

Procedures

According to Guideline for Prevention and Treatment of AIDS in China recommended by Chinese Medical Association,⁸ eligible participants in HIV-infected patients with virologic failure were: (1) HIV-positive (confirmed by ELISA and Western blot); (2) Individuals who have taken prescribed first-line ARV regimens for more than 6 months; (3) Individuals with viral load more than 1 000 copies/ml; (4) Individuals who continued to take suboptimal first-line ARV regimens or have discontinued first-line ARV regimens within 2 weeks. Exclusion criteria were: (1) Individuals who have taken prescribed first-line ARV regimens for less than 6 months; (2) Individuals who have taken second-line ARV regimens; (3) Individuals who have viral load less than 1 000 copies/ml; (4) Individuals who have discontinued ARV medications for more than 2 weeks.

Plasma samples were collected from HIV-infected individuals with first-line regimens every 6 months and HIV viral load was tested recommended by NFATP, individuals with viral load more than 1 000 copies/ml were selected to take HIV genotypic resistance testing.

Preparation of viral RNA templates

HIV RNA was extracted from the plasma using the QIAamp viral RNA kit and HIV reverse transcriptase-encoded gene was prepared to be amplified by PCR.

Reverse Transcriptional PCR

For detection of HIV reverse transcriptase-encoded gene, reverse transcriptional polymerase chain reaction (RT-PCR) was performed with a RT-PCR kit (PrimeScript[®] one step RT-PCR, Kit Ver. 2 Japan TATA Co.). Primers were designed based on reverse transcriptase-encoded gene sequence:

Outer primers: upstream: 5'-TTG GAA ATG TGG

AAA GGA AGG AC-3'; downstream: 5'-CTG TAT TTC TGC TAT TAA GTC TTT TGA TGG G-3'.

Inner primers: upstream: 5'-CAG AGC CAA CAG CCC CAC CA-3'; downstream: 5'-CTG CCA GTT CTA GCT CTG CTT C-3'.

Five µl HIV viral RNA was used as template and 20 µl premixed RT-PCR solution was taken for amplification, and PCR amplification was performed in a PCR System S1000 thermal cycler (Bio-Rad, CA, USA) in accordance with following protocols: reverse transcription at 50°C, 30 min; PCR amplification for 30 cycles with outer primers at 55°C, 30 s for denaturation, 72°C, 2.5 min for annealing and 72°C, 10 min for extension; followed by amplification for another 30 cycles with inner primers at 63°C, 30 s for denaturation, 72°C, 2.5 min for annealing and 72°C, 10 min for extension. The PCR products were electrophoresed with 1% agarose gel.

Sequencing of RT-PCR products and resistance analysis

The PCR products amplified with inner primers were sequenced by Shanghai Biotechnological Co. Ltd, and phylogenetic analysis was carried out with bio-software MEGA 4.0, and amplified sequence data were aligned with software Bioedit 7.0 to obtain the reverse transcriptase-encoded sequence, which would be further blasted with HIV resistance database in Stanford University (<http://hivdb.stanford.edu>).

RESULTS

Descriptive data in HIV-infected patients with virologic failure

In this study, 30 HIV-infected patients with virologic failure have been recruited in Beijing Ditan Hospital, Capital Medical University. Details of 30 HIV-infected patients with virologic failure were listed in Table 1. Mean age was 35±7 years; 28 were male. Average time on HAART was 29 months (SD = 16 months), 5 patients took HAART for less than 12 months, 16 patients for 12-36 months, and 9 patients for more than 36 months. All of recruited patients have taken first-line ARV regimens recommended by Guideline for Prevention and Treatment of AIDS in China.⁸

Prevalence of resistance to first-line regimens in HIV-infected patients with virologic failure

Total of 28 PCR products were amplified and

sequenced successfully in 30 serum samples of recruited HIV-infected patients with virologic failure. Resistance was found in 27 patients, which indicated that prevalence of resistance was 96%, mutations in NRT region were found in 26 patients (93%), while mutations in NNRT region existed in 27 patients (96%). In mutations leading to NRTIs resistance, single mutation M184V occurred in 6 patients, 2 or more mutations were selected in 20 patients, and total of 24 resistance mutations were developed in all recruited patients, in which M184V was the most common mutation and prevalence was 86% (24/28), including one case of M184V/I mutation (Figure 1). Other common mutation leading to NRTIs resistance were K70R (18%), D67G (14%), K70R (14%), A62V (14%) and K219E (14%).

In this study, it was found that thymidine analog mutations (TAMs) occurred in 14 patients and incidence was 50% (14/28), in which 1 TAMs was observed in 3 patients, 2 TAMs in 8 patients, 3 TAMs in 1 patients and 4 TAMs in 2 patients, respectively. We also found that M184V emerged in the presence of TAMs in 12 patients.

In mutations leading to NNRTIs resistance, single mutation occurred in 9 patients, while 2 or more mutations were observed in other patients. Total of 24 NNRTI mutations were selected in all recruited patients, in which Y181C and V179D were the most common mutations and prevalence were 43% (12/28) and 36% (10/28), respectively. Other common mutation leading to NNRTIs resistance were K103N (14%) and V179D (11%), respectively (Figure 2).

Except the above mutations on the reverse transcriptase gene leading to NNRTIs or NRTIs

resistance, PIs-specific minor mutations were also found in 5 patients, including A71T, Q58E, A71V, and N83D/N.

Genotype resistance in ARV regimens

In this study, intermediate or high-grade resistance to 3TC or FTC occurred in 89% of patients due to M184V mutation. Intermediate or high-grade resistance to ABC, AZT or d4T occurred in 30% of patients due to TAMs or TAMs in combination with M184V. Resistance to TDF occurred in 14% of patients due to K65R mutation, prevalence of which was lower than that occurred in AZT because most of patients took first-line ARV regimens contained AZT. Resistance to EFV or NVP occurred in 82% of patients and cross-resistance to ETR or RPV occurred in 50% of patients due to low-genetic barrier of NNRTIs. PIs-specific minor mutations were developed in 5 patients, which resulted in low-grade resistance to PIs (Figure 3).

DISCUSSION

In American Guideline for the Use of Antiretroviral Agents in HIV-1-infected Adults and Adolescents,³ HIV drug-resistance testing was recommended for HIV-infected individuals when they entered into care regardless of whether antiretroviral therapy was initiated or deferred; and in Guideline for Prevention and Treatment of AIDS in China,⁸ HIV drug-resistance testing was suggested in individuals with virologic failure after administering first-line antiretroviral regimens for more than 6 months, which helped to evaluate resistance and sensitivity of HIV

Table 1. Demographic characteristics in 30 HAART-experienced patients with virologic failure on first-line antiretroviral therapy in Beijing Ditan Hospital, Capital Medical University

Characteristics	Sample (Mean $\pm$ SD)
Age (years)	24-51 (35 $\pm$ 7)
Gender (cases)	Male (28 cases), female (2 cases)
Transmission routes (cases)	Sexual (26 cases), others (4 cases)
Baseline VL (copies/ml)	3.02-5.33 (4.03 $\pm$ 0.59)
Baseline CD4 levels (cells/ μ l)	88-529 (263 $\pm$ 111)
ARV regimens (cases)	AZT+3TC+NVP (18)
	D4T+3TC+NVP (6)
	D4T+3TC+EFV (3)
	AZT+3TC+EFV (2)
	TDF+3TC+EFV (1)
Months on HAART (Months)	6-67 (29 $\pm$ 16)

Figure 1. TAMs in HIV-infected individuals with M184 mutation.

Notes: 3TC-specific mutation, M184, often combines with TAMs in HAART-experienced patients with NRTIs resistance. TAMs and M184 mutation can result in NRTIs resistance through different mechanisms. M184 causes 3TC-specific resistance through sterical inhibition and incorporation of NRTIs is prevented in favor of dNTPs, while TAMs cause NRTIs resistance through phosphorolysis, which leads to the excision of the NRTIs already incorporated into the growing DNA chain. M184 mutation in combination with TAMs result in high resistance to NRTIs.

Figure 2. NRTIs and NNRTIs mutations in HIV-infected individuals administering first-line ART regimens.

Notes: Different NRTIs and NNRTIs resistance sites have been found in HAART-experienced patients with first-line ART regimens, AZT/d4T/TDF +3TC+EFV/NVP.

and guide to design an optimal second-line ARV regimen for these patients.

In this study, we recruited 30 cases of patients with virological failure, and reverse transcriptase-encoded gene in these serum samples was amplified, aligned

and comparatively analyzed with HIV resistance database, 137 mutations were found in these samples, which resulted in resistance to NRTIs or NNRTIs in 96% of recruited patients. It was reported that,⁹ among HAART-experienced patients with virologic

Figure 3. Percentage of resistance in NRTIs and NNRTIs in HIV-infected individuals.

Notes: Intermediate or high-grade resistance to 3TC or FTC occurred in 89% of patients due to M184V mutation. Intermediate or high-grade resistance to ABC, AZT or d4T occurred in 30% of patients due to TAMs or TAMs in combination with M184V. Resistance to TDF occurred in 14% of patients due to K65R mutation, prevalence of which was lower than that occurred in AZT because most of patients took first-line ARV regimens contained AZT. Resistance to EFV or NVP occurred in 82% of patients and cross-resistance to ETR or RPV occurred in 50% of patients due to low-genetic barrier of NNRTIs. PIs-specific minor mutations were developed in 5 patients, which resulted in low-grade resistance to PIs.

failure, more than one major mutation were found in 83.5% of patients, while dual or tripartite mutations occurred in 64.3% or 2.6% of patients, respectively. In this study, it was revealed that intermediate or high-grade resistance occurred in 89% of recruited patients, in which dual mutations occurred in 82% of patients. Second-line ARV regimens were used based on drug-resistance testing among these recruited patients, and no viral rebound was found after 48-week follow-up, which indicated that HIV drug-resistance testing was important to guide healthcare workers to design an optimal second-line ARV regimen for HAART-experienced individuals with virologic failure.

In this study, it was found that M184V mutation in reverse transcriptase was the most common mutation and occurred in the 86% of recruited patients. It was reported that M184V mutation would develop after lamivudine monotherapy for a few weeks, the M184V mutation did have advantages: not only did it improve the susceptibility of certain AZT-resistant HIV viruses in some patients, but also it impaired viral fitness.¹⁰ M184V mutation increased the susceptibility to AZT, d4T and TDF, while decreased the susceptibility to ddI and ABC. Keep lamivudine as part of ARV regimen despite M184V mutation was still sensible in order to conserve the M184V mutation and thus reduced the replicative capacities of HIV.

In this study, K65R mutation was found in 3 recruited patients. K65R mutation was rarely found before introduction of TDF, but K65R mutation was

primarily selected by TDF and led to an intermediate resistance to TDF, d4T, ddI, ABC and 3TC. K65R mutation decreased incorporation of NRTIs into the growing DNA chain and decreased the excision of NRTIs already incorporated into the DNA chain, which resulted in a decreased susceptibility to NRTIs but an increased susceptibility to AZT.¹¹ K65R mutation rarely emerged in the presence of TAMs because K65R and TAMs represented antagonistic resistance mechanisms.¹¹ In this study, it is found that K65R mutation emerged in presence of TAMs in 2 recruited patients due to side effects after administration of ddI and alteration to AZT, which resulted in high-grade resistance to AZT and compromised antiretroviral effects of second-line ARV regimens.

TAMs included the mutations in 41st, 67th, 70th, 210th, 215th and 219th amino acid in reverse transcriptase, which were primarily selected by thymidine analogs AZT and d4T, and resulted in cross-resistance to AZT or d4T. It was reported that prevalence of TAMs was 56% in HAART-experienced patients with virologic failure;¹² and prevalence was 80% in patients taking TDF+3TC+AZT for 48 weeks and then confirmed virologic failure;¹³ and there was more extensive genotypic resistance in three nucleotide analogs combinations than that in two NRTIs plus one NNRTI combinations.¹⁴ In this study, all the recruited patients were administered first-line ARV regimens, AZT/d4T+3TC+EFV/NVP,

and we found that prevalence of TAMs was 50%. Among 8 patients with intermediate or high-grade resistance to AZT, all of them had found TAMs, in which more than 3 TAMs occurred in 5 patients, and cross-resistance to d4T occurred in 7 patients, which indicated that the combination of certain TAMs can result in cross-resistance to AZT or d4T in this study.

M184V mutation in combination with TAMs indicated intermediate or high-grade resistance to 3TC, d4T and AZT at the same time. In this study, we found that M184V mutation in combination with TAMs occurred only in 6 patients (20%), which indicated that prevalence of this combination was uncommon in HIV-infected individuals with virologic failure. Q151M mutation was relatively uncommon, which alone led to intermediate resistance to AZT, ABC, ddI and d4T. Q151M combined with mutations at positions 75, 77 and 116 conferred high-grade resistance to AZT, ABC, ddI and d4T. T69 mutation conferred multidrug resistance to nucleoside analogs except for 3TC or FTC. In this study, we found that T69 or Q151 mutation combined with TAMs occurred in 2 patients, which led to high-grade resistance to all NRTIs.

It was reported that the prevalence of intermediate and high-grade resistance to AZT was more than 50% in Chinese HAART-experienced patients with virologic failure.¹⁵ We found that resistance to AZT occurred in 29% of recruited patients in this study, which was significantly lower than that occurred in Chinese HAART-experienced patients, it may be associated with periodical HIV viral load detection and timely alteration to ARV regimens after confirming virologic failure in Beijing Ditan Hospital. TDF was recommended as a choice in second-line ARV regimens in Guideline for Prevention and Treatment of AIDS in China,⁸ which may induce K65R mutation in spite of lower incidence.¹¹ K65R increased sensitivity to AZT and induced a resensitization to AZT, which indicated that current first-line ARV regimen, TDF+3TC+EFV, was a preferred choice in HAART-naïve patients, TDF can be changed to AZT if K65R mutation occurred in these patients.

In this study, NNRTIs resistance mutations have been found in all selected patients. The most common mutations were Y181C (43%) and G190A (36%), which coincided with some reports.¹⁶ Other common mutation sites found in our study were K103N(14%) and V179D (11%). NNRTIs were ARV drugs with

low-genetic barriers and NNRTIs resistance may occur individually or in combination. A single mutation can confer high-level resistance to one or more NNRTIs. In this study, we found that above single mutation, such as Y181C, G190A, K103N and V179D, conferred cross-resistance to NVP or EFV. ETR and RPV were second-generation NNRTIs and in vitro passage experiments suggested that resistance to ETR and RPV emerged more slowly than resistance to first-generation NNRTIs.¹⁷ Although no ETR or RPV was administered in these recruited patients, intermediate and high-grade resistance to ETR and RPV occurred in more than 50% of patients due to cross-resistance, which indicated that, in a patients with evidence of resistance to NRTIs, NNRTIs in first-line regimens should be discontinued promptly to reduce the risk of additional drug-resistance mutations to NNRTIs due to low-genetic barriers.

In this study, it was found that virologic failure may occur in long-term administration of first-line ARV regimens, and drug-resistance mutations can be found in these patients, which resulted in resistance to first-line ARV regimens. It's emphasized that HIV viral load assay was an important tool to guide healthcare workers to design an optimal second-line ARV regimens for HAART-experienced individuals with virologic failure.

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