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Study of HIV-1 Drug Resistance in Patients Receiving Free Antiretroviral Therapy in China*

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Abstract: To investigate the prevalence of drug-resistance mutations, resistance to antiretroviral drugs, and the subsequent virological response to therapy in treatment-naive and antiretroviral-treated patients infected with HIV/AIDS in Henan, China, a total of 431 plasma samples were collected in Queshan county between 2003 and 2004, from patients undergoing the antiretroviral regimen Zidovudine + Didanosine + Nevirapine (Azt+Ddi+Nvp). Personal information was collected by face to face interview. Viral load and genotypic drug resistance were tested. Drug resistance mutation data were obtained by analyzing patient-derived sequences through the HIVdb Program (http://hivdb.stanford.edu). Overall, 38.5% of treatment-naive patients had undetectable plasma viral load (VL), the rate significantly increased to 61.9% in 0 to 6 months treatment patients (mean 3 months) (P<0.005) but again significantly decrease to 38.6% in 6 to 12 months treatment patients (mean 9 months) (P<0.001) and 40.0% in patients receiving more than 12 months treatment (mean 16 months) (P<0.005). The prevalence of drug resistance in patients who had a detectable VL and available sequences were 7.0%, 48.6%, 70.8%, 72.3% in treatment-naïve, 0 to 6 months treatment, 6 to 12 months treatment, and treatment for greater than 12 months patients, respectively. No mutation associated with resistance to Protease inhibitor (PI) was detected in this study. Nucleoside RT inhibitor (NRTI) mutations always emerged after non-nucleoside RT inhibitor (NNRTI) mutations, and were only found in patients treated for more than 6 months, with a frequency less than 5%, with the exception of mutation T215Y (12.8%,

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6/47) which occurred in patients treated for more than 12 months. NNRTI mutations emerged quickly after therapy begun, and increased significantly in patients treated for more than 6 months (P<0.005), and the most frequent mutations were K103N, V106A, Y181C, G190A. There had been optimal viral suppression in patients undergoing treatment for less than 6 months in Queshan,Henan. The drug resistance strains were highly prevalent in antiretroviral-treated patients, and increased with the continuation of therapy, with many patients encountering virological failure after 6 months therapy.

Key words: Human Immunodeficiency Virus Type 1(HIV-1); Acquired Immunodeficiency syndrome (AIDS); Viral load; Antiretroviral therapy; Drug resistance

The introduction of highly active antiretroviral therapy (HAART) has markedly decreased mortality and morbidity in patients infected with HIV/AIDS Acquired Immunodeficiency syndrome) (11). However, HAART cannot completely eradicate HIV from the body, results in long-term toxicity and eventually leads to the emergence of drug-resistant HIV strains under the drug pressure *in vivo*, which was the main obstacle to the effectiveness of antiretroviral therapy (ART) (3). HAART therapy was introduced in Feb 2003 in Queshan, Henan, the first province to implements free ART in China under the policy of "Four Free and One Care". We performed drug resistance studies on several patients in this place to examine the prevalence of drugresistance mutations, resistance to antiretroviral drugs, and the subsequent virological response to therapy in HIV-1 infection.

MATERIALS AND METHODS

Study population

A total of 431 plasma samples were collected from patients infected with HIV/AIDS in Queshan County, Henan Province between 2003 and 2004, of which 104 samples were from treatment-naïve patients, 97 samples from patients undergoing treatment for less than 6 months (mean 3 months), 140 samples from patients undergoing treatment for 6 to 12 months (mean 9 months) and 90 samples from patients undergoing treatment for more than 12 months (mean 16 months). 97% of the patients were infected by paid plasma donation in the 1990s. All antiretroviral treated patients were under the regimen of Azt+Ddi+Nvp. All patients were informed consented and personal information was collected by face to face interview.

Viral load testing and $CD4^+$ cell count

Viral Load was tested with the Human Immunodeficiency Virus (HIV) Nucleic Acid Amplification (PCR) Fluorescent Detection Kit (PG, Shenzhen, China), the lower detection limit (LDL) is 500 copies/mL. The $CD4^+$ cells were counted by flow cytometry (Reagents and equipment from BD Co.)

RNA extraction, RT-PCR and sequencing

Viral RNA was extracted from 140μL plasma using the QIAamp Viral RNA Kit according to the manufacturer's protocol (Qiagen, Valencia, Calif.). The RNA template was then reverse transcribed to cDNA. From the cDNA template, nested-PCR was used to amplify a 1300 bp fragment of the *pol* gene comprising the entire protease-encoding region and first 300 codons of the RT-encoding sequences. After PCR, the products were purified using he Qiagen Gel Extraction Kit. The HIV-1 reverse transcriptase (RT) and genes

of protease were sequenced by the Tianjin biological chip company. The primers used for amplification and sequencing were previously reported (15).

Drug resistance analysis

After editing and assembly using Vector NTI (Version 10.0, Invitrogen Co.) and Bioedit (Version 7.0.5, downloaded on http://www.mbio.ncsu.edu/BioEdit/bioedit.html), the sequences were analyzed for genotypic antiretroviral resistance through the HIV db Program (Version 4.1.8, http://hivdb.Stanford.edu). The drug resistance profiles were analyzed by using genotypic and phenotypic interpretations defined by the Stanford HIV RT and Protease Sequence Database. This program identifies primary and secondary resistance mutations at specific codons in the Protease and RT regions and determines whether they confer resistance or sensitivity to certain NNRTIs, NRTIs, or PIs. The Stanford HIVdb software assigns drug resistance level as one of the following possibilities: susceptible (S), potential low-level resistance (P), low-level resistance (L), intermediate- level resistance (I), high-level resistance (H). In this study, we consider a patient with resistance as having mutations conferring L, I or H level resistance to one or more drugs. Patients without mutations or with drug resistance mutations conferring potential lowlevel resistance or susceptible to all drugs are considered to be currently susceptible to drug treatment or having no resistance.

RESULTS

$CD4^+$ cell count, viral load and correlated drug resistance

The results of $CD4^+$ cell count and measurement of viral load in untreated and treated groups are shown in Table 1. 38.5% of treatment-naïve patients had undetectable plasma viral load (VL), which signifycantly increased to 61.9% in 0 to 6 months treatment patients (mean 3 months) (χ^2=10.987, P=0.001), but again significantly decreased to 38.6% in 6 to 12 months treatment patients (mean 9 months) (χ^2= 12.444, P<0.001) and 40.0% (χ^2=8.926, P=0.003) in treatment more than 12 months patients (mean 16 months) (χ^2=8.248, P=0.004). With the increase of viral load in each group, the prevalence of drug resistance was getting higher and higher. The drug resistance rates increased with time of treatment in the same range of viral load.

Drug resistance mutations

The plasma samples with detectable viral load were tested for drug resistance genotypes. In the 241 samples

Table 1. Viral load and drug resistance analysis in each group

	Untreated group(N=104)	0-6 months therapy(N=97)		6-12 months therapy(N=140)		12- months therapy(N=90)	
	cases	cases	DR[b]	cases	DR	cases	DR
Viral load (log_{10} copies/mL)							
<LDL[a]	40	60		54		36	
>LDL&≤5	25	18	7(38.9%)	30	14(46.7%)	28	14(50%)
≥5	39	19	11(57.9%)	56	37(66.1%)	26	20(76.9%)
Mean viral load[c]	4.1±1.3	3.5±1.2		4.2±1.4		4.0±1.3	
Mean $CD4^+$ cell count (cells/μL)	364.8±209.1	405.6±280.4		408.0±252.9		363.3±279.3	

[a] <LDL (lower detection limit) indicates viral load is undetectable; >LDL&≤5 indicates viral load is detectable but less than 5 log_{10} copies/ml; ≥5 indicates viral load is no less than 5 log_{10} copies/ml; [b] DR is short for drug resistance; [c] Patients with undetectable viral load were assigned the LDL of viral load test in this study, 2.7 log_{10} copies/mL.

with detectable viral load, only 199 had corresponding sequences, so the genotyping results were limited to these samples. Within each patient group, there were large quantity of PI minor mutations, including types of L63P/S、A71V/T、V77I and I93L. Only one PI major mutation, V82I was found in the untreated group. The results are detailed in Table 2 and Table 3.

No mutation associated resistance to NRTIs was found in untreated and 0-6 months therapy groups. The mutation rates of resistance to NRTI were 8.3% and 19.0% in 6-12 months therapy group and greater

Table 2. Frequency of drug resistance mutations in each group

	Untreated group(N=43)	0-6 months therapy(N=37)	6-12 months therapy(N=72)	12- months therapy(N=47)
NRTI related mutations (%)				
M41L	0	0	0	1(2.1)
D67N	0	0	1(1.4)	1(2.1)
T69S_ST	0	0	1(1.4)	0
K70N	0	0	1(1.4)	0
K70R	0	0	0	2(4.3)
L74V	0	0	1(1.4)	0
V118I	0	0	0	1(2.1)
M184V	0	0	0	1(2.1)
L210W	0	0	0	2(4.3)
L210F	0	0	0	1(2.1)
T215F	0	0	1(1.4)	0
T215Y	0	0	1(1.4)	6(12.8)
K219N	0	0	0	1(2.1)
NNRTI related mutations (%)				
L100IV	0	0	1(1.4)	0
K101E	0	0	5(6.9)	2(4.3)
K101Q/R[a]	3(7.0)	0	1(1.4)	1(2.1)
K103N	2(4.7)	12(32.4)	38(52.8)	26(55.3)
K103S/E	0	0	1(1.4)	3(6.4)
V106A	0	4(10.8)	3(4.2)	1(2.1)
V106I[b]	2(4.7)	0	3(4.2)	2(4.3)
V108I	0	0	0(0.0)	1(2.1)
Y181C	1(2.3)	2(5.4)	6(8.3)	10(21.3)
Y188L	0	0	1(1.4)	0
G190A	0	3(8.1)	17(23.6)	11(23.4)
G190S	0	0	0	1(2.1)
F227L	0	1(2.7)	0	0
M230R	0	0	0	1(2.1)
K238T	0	0	2(2.8)	3(6.4)
K238R[c]	0	0	0	1(2.1)

[a]K101Q/R do not cause NNRT1 resistance; [b]V106I does not cause NNRT1 resistance; [c]K238R dose not cause NNRT1 resistance.

Table 3. Description of drug resistance mutations in each group

	Untreated group(N=43)	0-6 months therapy(N=37)	6-12 months therapy(N=72)	12- months therapy(N=47)
NRTI related mutations (%)	0	0	6(8.3%)	9(19.0%)
NNRTI related mutations (%)	8(18.6%)	18(48.6%)	54(75.0%)	36(76.6%)

than 12 months therapy group, respectively. The most common mutation was T215Y, with a rate of 12.8% found in the greater than 12 months therapy group. Other NRTI mutations were found at a lower frequency less than 5% in each group.

NNRTI mutations emerged quickly after therapy begun, and increased significantly in patients treated for more than 6 months (χ^2=9.646, *P*=0.002). In the untreated group, K101Q/R was more common than for the treated groups. The most frequent mutations were K103N, V106A, Y181C and G190A after therapy, and the rates of K103N, Y181C and G190A increased with the continuation of treatment. The frequency of K103N was 32.4% in patients treated for less than 6 months，and increased to 52.8% and 55.3% in 6-12 months therapy group and the greater than 12 months therapy group, respectively. Table 4 showed the mutation patterns of NNTRI in each group: in patients treated for less than 6 months there were mostly single mutations, while the strains with two or more NNRTI mutations increased with the continuation of therapy(χ^2=4.320, *P*=0.038).

Drug resistance analysis

The prevalence of drug resistance in patients who had detectable VL with sequences available were 7.0% (3/43), 48.6% (18/37), 70.8% (51/72) and 72.3% (34/47) respectively in untreated group, 0-6 months therapy group, 6-12 months therapy group and the greater than 12 months group, among which the rates increased dramatically in patients treated for more than 6 months (χ2=6.529, *P*=0.011). The rates of drug resistance in each group were a little lower than the mutation rates presented in Table 3, for some mutations do not reduce drug susceptibility. No patients with resistance to PIs were detected in this study. NRTI resistant strains were found only in patients treated for more than 6 months, with the rates of 7.0% (5/72) and 19.0% (9/47) in 6-12 months therapy group and the greater than 12 months therapy group (χ^2= 4.080, *P*=0.043), respectively. In the greater than 12 months therapy group, resistance to different NRTIs were all higher than that in the 6-12 months therapy group, among which the drugs with resistance rates more than 10% were Abacavir (Abc), Azt, Stavudine (D4t), Ddi and Tenofovir (Tdf), and high-level resistance to Lamivudine (3tc), Azt and Emtricitabine (Etc) were found at a low frequency (<5%). The rates of resistance to NNRTIs in treated patients were high, almost all were high-level resistance, and the rates increased significantly after 6 months therapy (χ^2= 9.646, *P*=0.002). Resistance to Delavirdine (Dlv) and Efavirenz (Efv) were slightly lower than Nvp. Resistance to both NRTIs and NNRTIs were found in patients treated for more than 6 months (Table 5).

For the Azt+Ddi+Nvp regimen under treatment, patients mainly presented resistance to Nvp, which increased significantly in patients treated for more

Table 4. Frequency of NNRTI mutations in treated patients

NO. of mutations	0-6 months therapy (N=37)			6-12 months therapy (N=72)			12- months therapy (N=47)		
	Cases	FA[a]	FM[b]	Cases	FA	FM	Cases	FA	FM
1	14	37.8%	77.8%	31	43.1%	57.4%	15	31.9%	41.7%
2	4	10.8%	22.2%	20	27.8%	37%	16	34.1%	44.4%
≥3	0	0.0%	0.0%	3	4.2%	5.6%	5	10.6%	13.8%
Total	18	48.6%	100%	54	75.0%	100%	36	76.6%	100%

[a] frequency in total patients. [b] frequency in patients with NNRTI mutations.

Table 5. Drug resistance analysis in each group

	NRTI		NNRTI		NRTI & NNRTI	
	Total DR[a]	H[b]	Total DR	H	Total DR	H
Untreated (N=43)	0	0	3(7.0%)	3(7.0%)	0	0
0-6 months (N=37)	0	0	18(48.6%)	18(48.6%)	0	0
6-12 months (N=72)	5(7.0%)	0	51(70.8%)	50(69.4%)	5(7.0%)	0
12- months (N=47)	9(19.0%)	3(6.4%)	34(72.3%)	34(72.3%)	9(19.0%)	3(6.4%)

[a] total drug resistance rate. [b] H indicates high-level resistance to drugs.

than 6 months (χ^2=6.529, *P*=0.011). Resistance to Azt and Ddi was only present in patients with Nvp resistance and were seen after 6 months of therapy. Resistance to all three drugs was 4.2% (3/72) and 12.8% (6/47) in patients treated for 6 to 12 months and treated for more than 12 months, respectively (Fig.1).

DISCUSSION

Eradication of HIV infection cannot be achieved with available antiretroviral regimens. This is chiefly because the pool of latently infected CD4+ T cells is established during the earliest stages of acute HIV infection and persists with a long half-life, even with prolonged suppression of plasma viremia (4, 10).

Therefore, once the decision is made to initiate therapy, the primary goals of antiretroviral therapy are to suppress viremia to less than detection limits,

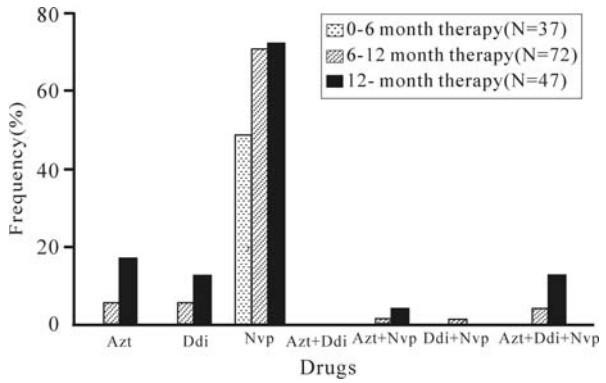

Fig.1. Analysis of resistance to the drugs under therapy. Azf: Anfirefroviral regimen zidovuding. Ddi: Didanosine. Nvp: Nevirapine.

prevent further immune deterioration, avoid HIV-associated morbidity and mortality and improve quality of life (10). The U.S. Department of Health and Human Services (DHHS) Panel on Antiretroviral Guidelines for Adults and Adolescents recommends that an initial regimen contain two NRTIs and either a NNRTI or a ritonavir-boosted or unboosted PI (10). The regimen of our study population is two NRTIs (Azt and Ddi) and one NNRTI (Nvp).

Viral load is critical for evaluating response to therapy. Virological failure during treatment can be defined as a confirmed HIV RNA level >400 copies/mL after 24 weeks, >50 copies/mL after 48 weeks, or a repeated HIV RNA level >400 copies/mL after prior suppression of viremia to <400 copies/mL (10). In our study, we found that there had been optimal viral suppression in patients treated for less than 6 months compared to treatment-naïve patients, but in patients treated for more than 6 months, more patients encountered virologic failure. This was co-related with the dramatic increase of drug resistant strains especially the rates of resistance to both of the two classes of drugs in patients with long-term treatment. The development of drug resistance to the NRTIs, NNRTIs or PIs, is both a cause and a result of virologic treatment failure with incomplete virus suppression (7). Our study results also showed that with the increase of viral load, the prevalence of drug resistance was getting higher and higher. Besides,

poor adherence to ART is also a very important reason for treatment failure. Our past study in this area proved that patients here had poor drug adherence (5). Li JY *et al* also showed that poor adherence to therapy was believed to be the main reason for the emergence and prevalence of drug resistant HIV strains (9).

HIV, like many other retroviruses, is prone to the incorporation of mutations during replication at a rate of 3×10^{-5} mutations/nucleotide. Every possible mutation along the genome can occur 10^4 or more times daily. With the rapid development and outgrowth of mutant resistant virus, viral replication can lead to the selection of drug resistant isolates and eventual failure of the therapeutic regimen (1). Our data showed that the prevalence of drug resistance in treatment-naive patients was still at a low level compared to a nationwide study in 2002 (16), and indicated that there had been low-level transmission of drug resistance since large-scale implementation of free HAART in China. Drug resistance emerged quickly after treatment begun and increased significantly after 6 month of therapy, the rate of resistance reached 72.3% in patients treated for more than 12 months with detectable viral load. Richman D *et al* also reported that the prevalence of drug resistance was 75% in the U.S. (12), much the same level as ours.

When the genotyping was performed, we found that some PI minor mutations like L63P/S, A71V/T, V77I and I93L was commonly present in both untreated and treated patients yet did not cause resistance to PIs, further research should be done to determine whether these existing mutations affect the using of PIs in the future. One PI major mutation, V82I, was found in a treatment-naïve patient. This mutation infrequently emerges in untreated patients but did not cause resistance to any PIs. So, all study patients were susceptible to PIs, for currently no PIs were under treatment.

Mutations associated with resistance to NRTIs emerged after resistance to NNRTIs. T215Y was the most frequent NRTI mutation, which causes Azt and D4t resistance and limits the effectiveness of Abc, Ddi, and Tdf, and was most commonly seen in patients receiving two NRTIs (14). Multi-NRTI resistance mutations, also known as nucleoside-associated mutations (NAMs), are associated with resistance to numerous NRTIs. The M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E are known as thymidine-associated mutations (TAMs). TAMs are a subset of NAMs that are selected by the thymidine analogues zidovudine and stavudine and are associated with cross-resistance to all NRTIs currently approved by the U.S. FDA (6). In our study, L210W are always observed in tandem with T215Y, and only seen in patients treated for more than 12 months. Two samples had three TAMs, one with M41L, L210W and T215Y and another with D67N, K70R and T215Y; both presented high-level resistance to Azt, intermediate-level resistance to Ddi and caused cross resistance to Abc, D4t, and Tdf. Mutations associated with resistance to NNRTIs emerged quickly after therapy begun. One single NNRTI mutation could cause high-level resistance to one or more NNRTIs (14). In our study, K103N was the most frequent mutation, which caused high-level resistance to all currently approved NNRTIs, followed by V106A, Y181C and G190A. It is reported that Y181C increases susceptibility to Azt (13), the increasing frequency of this mutation with time might have some positive effect on the regimen.

For the Azt+Ddi+Nvp regimen in this study, resistance to Nvp was at a very high level quickly after treatment begun, resistance to Azt or Ddi were at a relatively low level and only presented in patients treated for more than 6 months. This result was much the same as Conway B's study with the same regimen (2). Our results showed that resistance to Azt or Ddi

was only present in patients with Nvp resistance; this may because of the high level Nvp resistance rates, or the presence of some additional unknown mechanism.

In summary, emergence of drug resistance strains and other factors such as poor adherence to regimen and bad patient management had led to high rate of virologic failure of long-term treatment. For patients with insufficient viral suppression, whether they should change regimen is a complex problem. Frequently changing of drugs would limit the following option of drugs (8) and currently there are few free optional drugs. So improving management and adherence is very important for patients under treatment to maximally reduce emergence of drug resistance, ensuring durable suppression of viral load.

Abbreviations in this paper

HIV-1: Human Immunodeficiency Virus Type 1. HAART: Highly active antiretroviral therapy. ART: Antiretroviral therapy. AIDS: Acquired Immunodeficiency syndrome. RT: Reverse transcriptase. PI: Protease inhibitor. NRTI: Nucleoside RT inhibitor. NNRTI: Non-nucleoside RT inhibitor. VL: Viral load. Azt: Zidovudine. Ddi: Didanosine. Nvp: Nevirapine. D4t: Stavudine. 3tc: Lamivudine. Abc: Abacavir. Etc: Emtricitabine. Tdf: Tenofovir. Dlv: Delavirdine. Efv: Efavirenz.

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