Iranian Journal of Clinical Infectious Diseases
2008;3(4):215-219

BRIEF REPORT

Genotypic correlation of a virologic response to lamivudine, stavudine and nevirapine in patients for whom combination therapy had failed

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ABSTRACT

Background: Resistance is the consequence of mutations that emerge in the viral proteins targeted by antiretroviral agents. Thus, we focused our attention on mutations in HIV-1 reverse transcriptase to define their association with specific NRTIs and NRTI resistance mutations at therapeutic failure.

Patients and methods: The study population included 5 Iranian HIV-positive patients referring to Counseling Behavioral Modification Center in Shiraz who received a combination of antiretroviral therapy (lamivudine, stavudine and nevirapine). PBMC DNA was isolated from blood and PCR was performed to produce a 1200 bp amplicon and resolved by electrophoresis on a 0.7% agarose TBE gel, visualized with ethidium bromide. PCR products from HIV-1-infected patients were cloned into pCR2.1TOPO, then sequenced. Finally, sequence data were analyzed.

Results: Results showed drug resistance in 2 patients, of whom one had NNRTI resistance mutations (M230G, L234R and K238H) and other had both NRTI (V75M) and NNRTI (F227L) resistance mutations.

Conclusion: Confirmation of genetic resistance in HIV-positive patients who show therapy failure can help physicians to change their drug regime in order to achieve better outcome.

Keywords: *Human immunodeficiency virus (HIV), Drug resistance, Nucleoside reverse transcriptase inhibitor (NRTI),Nonnucleoside reverse transcriptase inhibitor (NNRTI).*
(Iranian Journal of Clinical Infectious Diseases 2008;3(4):215-219).

INTRODUCTION

The use of combinations of antiretroviral drugs has proven remarkably effective in controlling the progression of human immunodeficiency virus (HIV) disease and prolonging survival (1), but these benefits can be compromised by the development of drug resistance (2,3). Resistance is the consequence of mutations that emerge in the viral proteins targeted by antiretroviral agents. In the United States, as many as 50 percent of patients receiving antiretroviral therapy are infected with viruses that express resistance to at least one of the available antiretroviral drugs (4).

During its spread among humans, human immunodeficiency virus type 1 (HIV-1) has developed an extraordinary degree of genetic diversity, mainly due to the intrinsic inability of HIV-1 reverse transcriptase (RT) to carry out proofreading of DNA during replication (5) and

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exacerbated by the high rate of viral replication in vivo (109 viral particles produced daily). Among the different areas of the viral genome, the pol gene, encoding enzymes such as reverse transcriptase and protease, is subjected not only to natural evolutionary forces but also to selective pressure imposed by pharmacological treatment (6). The HIV-1 reverse transcriptase enzyme is responsible for the conversion of the single-stranded RNA genome into a double-stranded DNA that is later integrated into host genomic DNA (7,8). Owing to its pivotal role in the HIV-1 life cycle, the reverse transcriptase represents an attractive target for antiviral therapy. To April 2006 (6), 11 out of 21 compounds approved for the treatment of HIV-1 infection were reverse transcriptase inhibitors. In particular, they consist of the group of seven nucleoside analogue reverse transcriptase inhibitors (NRTIs; zidovudine, stavudine, lamivudine, didanosine, abacavir, zalcitabine, and emtricitabine), one acyclic nucleoside monophosphate (tenofovir, generally considered in the class of NRTIs), and three nonnucleoside analogue reverse transcriptase inhibitors (NNRTIs; nevirapine, efavirenz, and delavirdine). When antiviral therapy fails to be fully suppressive, new viral variants can emerge, allowing HIV-1 to escape from reverse transcriptase inhibitors by accumulating mutations, either alone or in multiple clusters, which affect the long-term therapy targeting reverse transcriptase (9-12). Several mutations or groups of mutations in HIV-1 reverse transcriptase can promote resistance by selectively impairing the ability of the enzyme to incorporate the nucleoside analogue into DNA. To date, mutations at 61 residues in HIV-1 reverse transcriptase have been related to treatment with the experimentally tested reverse transcriptase inhibitors (6). Of these, 18 sites are involved in resistance to the eight currently approved NRTIs and 16 sites are involved in resistance to the three currently approved NNRTIs.

Since resistance to reverse transcriptase inhibitors is a very complex phenomenon, it is conceivable that more mutations than currently known are involved in the development of drug resistance and therefore lead to therapeutic failure. Studies have shown that patients whose physicians have access to drug resistance data, particularly genotypic resistance data, respond better to therapy than control patients whose physicians do not have access to these assays. Thus, we focused on mutations in HIV-1 reverse transcriptase to confirm their association with specific NRTIs and NRTI resistance mutations with therapeutic failure.

PATIENTS and METHODS

The study population included 5 Iranian HIV-positive patients referring to Counseling Behavioral Modification Center in Shiraz who received a combination of antiretroviral therapy (lamivudine, stavudine and nevirapine) between January 2006 and February 2007. They aged 24-56 years. CD4 count did not increase in 2 patients. Averagely, 55 weeks following the therapy, blood samples were drawn. Then, PBMC DNA was isolated using a QIAGEN Blood Mini kit. First round PCR (polymerase chain reaction) was performed with primers PRA and IBR1. A second round of PCR was achieved with primers PRB and IBR2 to produce a 1200-bp amplicon. In the 1st round PCR, each cycle consisted of hot start for 5 minutes at 94ºC, denaturation for 20 seconds at 94ºC, annealing for 20 seconds at 55ºC, extension for 2 minutes at 72ºC and final extension for 7 minutes at 72ºC (35 cycles). In the 2nd round PCR (Nested PCR), each cycle consisted of hot start for 5 minutes at 94ºC, denaturation for 20 seconds at 94ºC, annealing for 20 seconds at 52ºC and extension for 1 minute at 72ºC and final extension for 5 minutes at 72ºC (35 cycles). The sequences of PCR primers are represented in table 1.

PCR cleanup was performed with a QIA quick PCR purification kit (QIAGEN). The purified

second-round PCR products were resolved by electrophoresis on a 0.7% agarose TBE (Tris-borate-EDTA) gel, visualized with ethidium bromide.

Table 1. The sequences of PCR primers

PCR	primers	Sequence (5'-3')
1st round	PRA	CCTAGGAAAAAGGGCTGTTGGAAATGTGG
	IBR1	AAATGATGACAGCATGTCAGGGAGT
2nd round	PRB	ACTGAGAGACAGGCTAATTTTTTAGGGA
	IBR2	CAAAGGAATGGAGGTTCTTTCTGATG

Clonal analysis was achieved for detection of drug resistance mutations in the HIV pol gene. The PCR products from HIV-1-infected patients were inserted into pCR2.1TOPO , a TA cloning vector (Invitrogen, USA) and transformed into Escherichia coli top10f' cells, according to the manufacturer's instructions. Plasmid purification was performed by QIAGEN Spin Mini kit. Interest plasmids were then sequenced by Sinnagen Co, Tehran, Iran. Sequence data were analyzed with HIV database, a program interprets user-entered mutations to infer the level of resistance to inhibitors of the reverse transcriptase and the protease inhibitors.

RESULTS

Amplification of pol gene: Pol gene was amplified in two steps, standard PCR with PRA, IBR1 and Nested-PCR with PRB primers. A non-specific bond observed above interested bond. Therefore, 100µl of PCR product prepared and cleaned up then ran on agarose gel 0.7% (Fig. 1).

TA-cloning of pol fragments: Initially selection was performed with white and blue method. White clones were removed from agar plate and subcultured in LB broth (5 ml tubes). Then, extracted recombinant plasmids were digesting with EcoRI enzyme that has restriction sites in flanking pol gene. Confirmed plasmids were sequenced and resulted data analyzed with HIV database (Fig. 2).

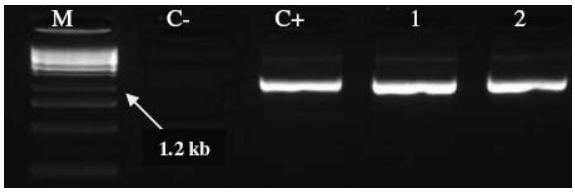

Figure 1. PCR result of samples. M (1 kb ladder), C- (without DNA), C+ (known sample) and samples 1, 2.

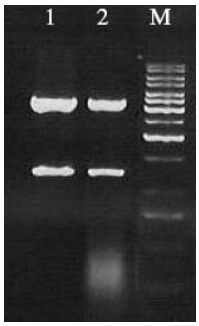

Figure 2. Detection of recombinant plasmids (containing pol gene) through digestion with restriction enzyme (EcoRI). M (1 kb ladder) and 1, 2 (samples).

HIV database analysis: Data analyses showed that there are no insertions or deletions in studied RT genes. However, subtype and similarity to closed reference isolates were: Sample 1 (RT: D (89.5%), sample 2 (RT: D (90.9%).

RT Comments about sample 1: Sample 1 has not NRTI resistance mutations but has NNRTI resistance mutations (M230G, L234R and K238H). These mutations are not well known and according to HIV database, G, R and H are atypical mutations at 230, 234 and 238 codons, respectively.

Table 2. RT resistance mutations

Sample	NRTI resistance mutation	NNRTI resistance mutation	RT other mutation
1	None	M230G, L234R, K238H	E6D, K11R, V21A, V35T, T39A, V60I, K101R, D121Y, K122E,D123E, I135R, N175Y, D177E, V179I, Q207A, R211K, G231E, Y232F, E233C, H235Y, D237
2	V75M	F227L	E6D, V8I, I31L, V35T, T39A, I50T, V60I, D121H, K122G, I135V,Q145R, D177E, V179I, T200I, Q207A, R211K, L228I

NRTI: Nucleoside reverse transcriptase inhibitor,
NNRTI: Nonnucleoside reverse transcriptase inhibitor

Table 3. Susceptibility of sample 2 to antiretroviral drugs

NRTI		**NNRTI**	
3TC	Susceptible	DLV	Susceptible
ABC	Susceptible	EFV	Susceptible
AZT	Susceptible	NVP	Susceptible
D4T	Susceptible		
DDI	Susceptible		
FTC	Susceptible		
TDF	Susceptible		

NRTI: Nucleoside reverse transcriptase inhibitor, **NNRTI**: Nonnucleoside reverse transcriptase inhibitor, **3TC**: lamivudine, **ABC**: abacavir, **AZT**: zidovudine, **d4T**: stavudine, **ddI**: didanosine, **TDF**: tenofovir, **FTC**: emtricitabine, **NVP**: nevirapine, **DLV**: delavirdine, **EFV**: efavirenz

Table 4. Susceptibility of sample 1 to antiretroviral drugs

NRTI		**NNRTI**	
3TC	Susceptible	DLV	Susceptible
ABC	Susceptible	EFV	Susceptible
AZT	Susceptible	NVP	Low-level resistance
D4T	Low-level resistance		
DDI	Potential low-level resistance		
FTC	Susceptible		
TDF	Susceptible		

NRTI: Nucleoside reverse transcriptase inhibitor, **NNRTI**: Nonnucleoside reverse transcriptase inhibitor, **3TC**: lamivudine, **ABC**: abacavir, **AZT**: zidovudine, **d4T**: stavudine, **ddI**: didanosine, **TDF**: tenofovir, **FTC**: emtricitabine, **NVP**: nevirapine, **DLV**: delavirdine, **EFV**: efavirenz

DISCUSSION

Three types of antiretroviral drugs (ARVs) are now used for the treatment of HIV-1 infections, but only reverse transcriptase (RT) inhibitors are readily available to the vast majority of HIV-1-infected individuals in the developing world. The treatment regimen of choice is a combination of a nonnucleoside RT inhibitor (almost exclusively nevirapine [NVP]) and two nucleoside RT inhibitors, i.e., zidovudine (AZT) or stavudine plus lamivudine or didanosine (16). In our country, these drugs have been used by medical centers, such as Counseling Behavioral Modification Center in Shiraz.

One of the main difficulties of patients who receive antiretroviral drugs is their resistance to these regimens. Meanwhile, unsuccessful therapy and resistance are not presented with characteristic clinical symptoms or CD4+ count. Recently, sequencing of pol gene has shown proving results. In deed, both NRTI and NNRTI resistance mutations have been detected with this approach. Some of the mutations such as V75M (NRTI resistance mutation) and F227L (NNRTI resistance mutation) despite M230G, L234R and K238H (NNRTI resistance mutations) have been reported previously (2,4,5,10-16). V75T/M/A is NRTI-selected mutation that occur in 2.1% (M) and 0.6% (A) of subjects receiving NRTIs and also appear to contribute to stavudine resistance (13,14). They cause stavudine and possibly didanosine resistance. F227L augments nevirapine resistance when present with V106A but does not cause resistance on its own or affect other NNRTIs (14,15).

Data analysis showed that these patients had different susceptibilities to different antiretroviral drugs. Therefore, knowledge of physician towards patient's susceptibility to antiretroviral drugs before therapy commencement may help him in prescribing the suitable drug regimens. However, availability of genotypic resistance data following an unsuccessful therapy makes them to change patient’s drug.

ACKNOWLEDGEMENT

We thank Ahmad Adeli, Elham Saboori, Hosna Gomari, Rezvan Esmaili, and Rosita Edalat (Pasteur Institute of Iran) and Naseri, Ansari, Davarpanah (Counseling Behavioral Modification Center in Shiraz for their helps.

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