

# Drug resistance profile and subtyping of HIV-1 RT gene among Iranian under-treatment patients

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## Abstract

Identification of drug resistant mutations is important in the management of HIV-1 infected patients. The aim of the current study was to evaluate drug resistance profile of RT gene and assess subtype among HIV-1 circulating strains and intensification of physician's options for the best therapy. HIV-1 RNA of 25 samples was extracted from plasma and RT Nested-PCR was performed and the final products were sequenced and phylogenetically analyzed. Stanford HIV drug resistance sequence database was used for interpretation of the data. The results of phylogenetic analysis showed subtypes A1 and B in 14 (58%) and 10 (42%) patients respectively. Of the 24 patients, 23 (95%) had resistance to NRTIs, 8 individuals (32%) to NNRTIs and one patient was susceptible to NRTIs as well as NNRTIs. The drug resistance interpretation in this study showed: 87.7% susceptible for AZT, 70.8% susceptible, and 25% high-level resistance for 3TC, 87.7% susceptible for TDF, 29.1% high-level resistance for NVP and 70.8% susceptible and 25% high-level resistance for EFV. Our data suggests that probably, the use of 2 NRTIs plus 1 protease inhibitor (PI) regimen is more effective than 2 NRTIs plus 1 NNRTI regimen in Iranian patients that use 2 NRTI plus NNRTI regimen and also continuous surveillance should be performed to evaluate resistance patterns for more effective therapeutic approaches.

**Keywords:** HIV-1; Drug resistance; Phylogeny; Iran

## 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; however, these benefits can be compromised by the development of drug resistance phenomenon (Francois *et al.*, 2004).

The main factors associated with treatment failure are the patient's inadequate adherence to therapy, incorrectly prescribed drugs (Palma *et al.*, 2007), and decrease in the pharmacological activity of antiretroviral agents as well as emergence of drug resistance associated mutations (Coffin, 1995). Whenever the virus is able to continue replication in the presence of drugs, drug-resistant variants emerge, as a result, the response to therapy is decreased (Kim *et al.*, 2006). Drug resistance is the inevitable consequence of incomplete suppression of HIV replication (Clark *et al.*, 2005). It has been shown that HIV is able to develop resistance to all currently used antiretroviral drugs (Van Vaerenbergh, 2007).

The evolution of HIV-1 drug resistance within an individual, depends on the generation of genetic variation (Clark *et al.*, 2005; Shafer *et al.*, 2000) and on the selection of drug-resistant variants during drug therapy. HIV-1 genetic variability is caused by the inability of HIV-1 RT to proofread nucleotide sequences during replication (Shafer *et al.*, 2000). Resistant viruses can emerge continuously and are transmitted causing a new public health concern. Some studies report very different rates of transmission of drug-resistant viruses, ranging from 20% in North America (Ristig *et al.*, 2002) to less than 10% in Europe (Chen *et al.*, 2007; Babic *et al.*, 2006).

The HIV-1 genotyping resistance test (GRT) has been widely used to monitor the antiretroviral treatment on HIV-1 patients (Hirsch *et al.*, 2000). GRT

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results would be an important factor for Highly Active Antiretroviral Therapy (HAART) regimen selection (Snoeck *et al.*, 2002). The aim of this study was to determine drug resistances profile of RT gene and assessing subtype among HIV-1 strains and intensification of physician's options for the more effective therapy.

## METHODS

**Sample collection:** In this cross-sectional study, 25 antiretroviral under treatment HIV-infected patients admitted to Infectious Disease Division of the Imam Khomeini Hospital for HIV-related illnesses. All patients received antiretroviral treatment for at least 1 year. The therapy regimen contained one non-nucleoside reverse transcriptase inhibitor (NNRTI) in combination with two nucleoside reverse transcriptase inhibitor (NRTIs). The mean CD4 cell count was  $197 \pm 8$  (38-274) cells/ml. Blood samples were collected in sterile EDTA-containing tubes and the plasma was separated and stored at  $-80^{\circ}\text{C}$ . Ethical approval was obtained from collecting center and Medical Research Ethics Committee, Tarbiat Modares University. Informed consent was taken from the patients before sample collection.

**HIV RNA extraction and cDNA synthesis:** Total RNA was extracted from 140  $\mu\text{l}$  of each plasma sample using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Following RNA viral denaturation at  $70^{\circ}\text{C}$  for 10 min, cDNA synthesis was performed at  $42^{\circ}\text{C}$  for 60 min, using 10 U of M-MuLV reverse transcriptase (fermentas) and 1 mM of antisense outer primers (Table 1). Plus 1.0 mM dNTP, 10 U RNase inhibitor (fermentas).

**Nested PCR amplification:** First-round PCR was performed using 3.5  $\mu\text{l}$  cDNA, 1X PCR Buffer, 0.2  $\mu\text{l}$  of 10 mM dNTPs, 0.1U of 5U Pfu polymerase, 1.5  $\mu\text{l}$  50

mM  $\text{MgSO}_4$ , 0.3 mM of 12.5 pico Mol/ $\mu\text{l}$  primer solution containing outer primers (Table 1) and 17  $\mu\text{l}$  of Double Distilled Water. Briefly, the amplification profile consisted of denaturation at  $95^{\circ}\text{C}$  for 5 min, followed by 30 cycles of denaturation at  $94^{\circ}\text{C}$  for 50 s, annealing at  $55^{\circ}\text{C}$  for 40 s, extension at  $72^{\circ}\text{C}$  for 55 s, followed by a final extension phase at  $72^{\circ}\text{C}$  for 3 min.

An aliquot (about 25  $\mu\text{l}$ ) of the primary PCR products was used for 35 cycles of nested PCR as follows; initial denaturation at  $95^{\circ}\text{C}$  for 5 min, 35 cycles of denaturation at  $94^{\circ}\text{C}$  for 50 s, annealing at  $58^{\circ}\text{C}$  for 40 s, and polymerisation at  $72^{\circ}\text{C}$  for 50 s, with a final elongation at  $72^{\circ}\text{C}$  for 3 min. An Eppendorf Gradient PCR system thermal cycler was used for all PCR reactions. The results were checked by electrophoresis of the nested PCR products on 2% agarose gels and visualized with ethidium bromide under UV light.

**Purification and DNA sequencing:** The PCR products (Table 1) were purified by Gel Purification kit (Bioneer, Global Genomics Partner), according to manufacturer's instructions and sequenced on both strands (bi-directionally) by the dideoxy chain termination method (ABI PRISM 3700 DNA analyzer automated DNA Sequencer, Applied Biosystem, Foster city, CA, USA).

**Drug resistance analysis and interpretation:** The RT sequences were multiple aligned by BioEdit software (version 5.0.6) and were analyzed through the Stanford University HIV Drug Resistance Database HIVdb program (version 4.2.6 [<http://hivdb.stanford.edu>]) for genotypic resistance interpretation (Gallant *et al.*, 2006; Campbell *et al.*, 2005). Finally drug resistance-associated mutations in RT gene were defined by the Stanford HIVdb version 4.2.6.

**Phylogenetics analysis:** The *pol* region has been identified as a reliable region for HIV-1 subtyping before (Pistello *et al.*, 2004; Njouom *et al.*, 2003). The sequences were aligned with a set of reference sequences of group M subtypes (A-K), CRFs, group O

**Table 1.** The sequence of primer pairs used for first (RTF1, RTR1) and second round (RTF2, RTR2) amplification, position and fragment length of PCR reaction.

Primer	Sequence	Position	Product Length
RTF1	5' AGTAGGACCTACACCTGTCAA 3'		
RTR1	5' TGTTAGTGCTTTGGTTCCCCT 3'	2096 to 3056	960 bp
RTF2	5' ATGGCCCAAAGGTTAAACAATGG 3'		
RTR2	5' TTCTGTATATCATTGACAGTCCAG 3'	2189 to 2929	740 bp

and Cpx, available at the Los Alamos Database (<http://hiv-web.lanl.gov>) and NCBI using ClustalW software. Reference sequences used for phylogenetic analysis were as follows:

subtype A1: SE7253,92UG037; subtype A2: 94CY017, 97CDKTB48; subtype B: HXB2, K02007, K03455, M17449; subtype C: 92BR025, ETH2220; subtype D: U88822, U88824, M27323; subtype F: VI850, 93BR020, MP411; subtype G: 92NG083, SE6165; subtype H: VI997, 90CR056; subtype J: SE9280; subtype K: MP535, EQTB11; subtype AB: AF193227, AF193275; subtype AE: AF197338, AF197340; subtype BC: AX149771; subtype AG: L39106; subtype CPX: AJ288981, AF049337; subtype O: L20571.

Phylogenetic trees were reconstructed using a Neighbor-Joining Method (NJ) with Molecular Evolutionary Genetics Analysis (MEGA) software, version 4. The reliability of the branching order was estimated by performing 1000 times bootstrap replicates (Sarrami-Forooshani *et al.*, 2006).

## RESULTS

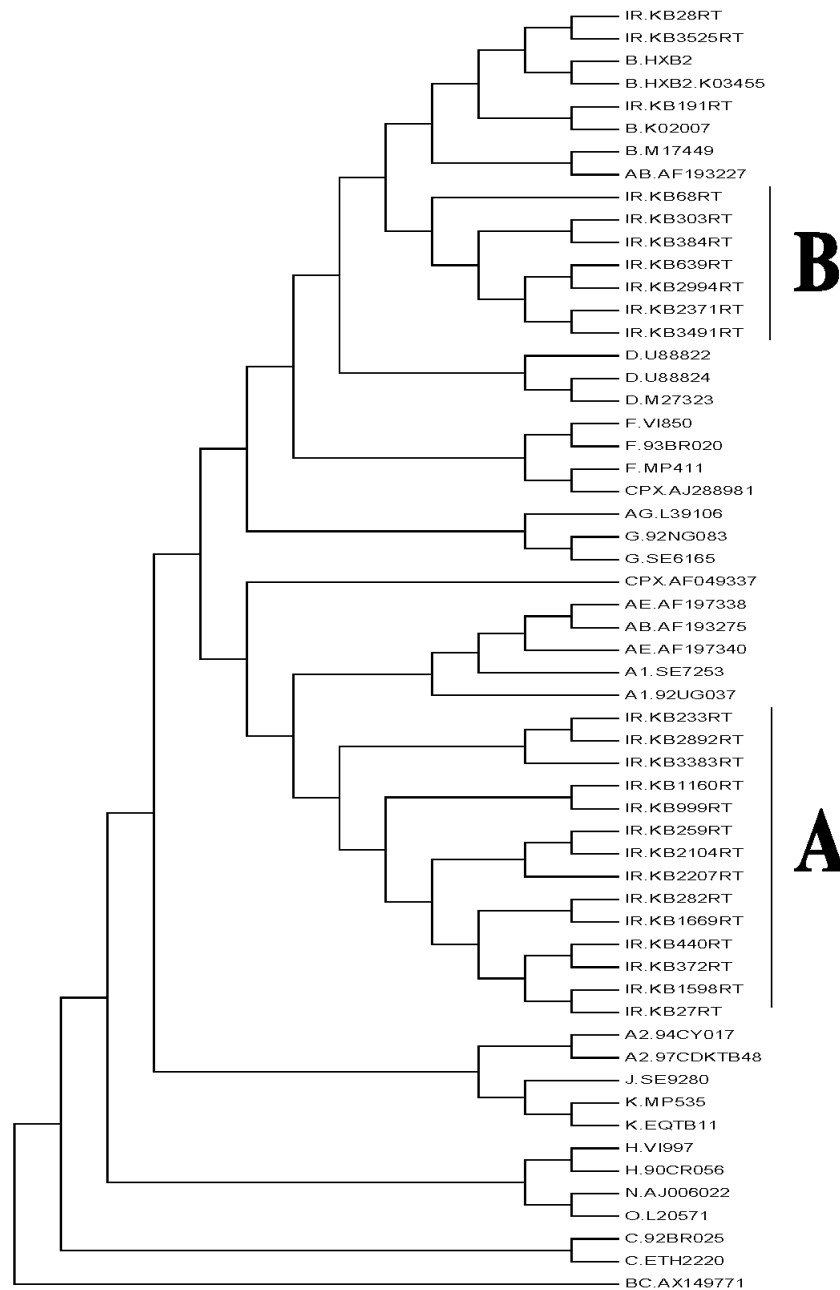
Among the 25 diagnosed HIV-1 infected patients enrolled in the study, 21 (84%) were males and 4 (16%) were females. Their mean age was 39 years. With respect to the mode of infection, as summarized in Table 2, 14 patients (56%) reported intravenous drug users, 7 patients (28%) reported hemophiliacs, and 4 patients (16%) had sexual contacts. Majority of patients (n= 17) were using the Lamivudine (3TC) plus Tenofovir (TDF) plus Efavirenz (EFV) regimen. In five patients, the regimen was Zidovudine (ZDV) plus 3TC plus EFV and in the two other patients the regimen was ZDV, 3TC, Nevirapin (NVP). All patients received antiretroviral treatment for at least 1 year.

Sequencing of the 25 RT amplicons gave suitable sequence data for all except one sample that failed to sequence. The results of phylogenetic analysis (Fig. 1) showed subtypes A1 and B in 14 (58%) and 10 (42%) patients respectively. The results also showed that the subtype A1 is the most prevalent in HIV infected

**Table 2.** Demographic information, risk group, subtype and drug resistance of the patients enrolled in the study.

ID	Sex	Age (Years)	Time of 1st HIV Test	Risk Group1	Subtype	Drug Resistance2
IR.KB1RT	Male	55	02-12-1995	Hemophiliacs	A	3TC(S), TDF(S), EFV(S)
IR.KB2RT	Female	47	02-12-1995	Homosexual	B	3TC(H), TDF(I), EFV(H)
IR.KB3RT	Male	33	03-05-1986	Hemophiliacs	B	3TC(H), TDF(S), EFV(S)
IR.KB4RT	Male	35	Unknown	Hemophiliacs	B	3TC(H), TDF(I), EFV(H)
IR.KB5RT	Male	37	Unknown	IVDU	A	AZT(S), 3TC(H), NVP(H)
IR.KB6RT	Male	45	15-08-2004	IVDU	A	TDF(S), 3TC(S), EFV(S)
IR.KB7RT	Male	33	07-10-2004	IVDU	A	TDF(S), 3TC(H), EFV(H)
IR.KB8RT	Male	38	14-11-2004	IVDU	B	TDF(S), 3TC(S), EFV(S)
IR.KB9RT	Male	46	17-12-2004	IVDU	A	AZT(H), 3TC(S), NVP(H)
IR.KB10RT	Male	34	11-01-2005	IVDU	B	3TC(S), TDF(S), EFV(S)
IR.KB11RT	Male	48	12-09-2005	IVDU	A	TDF(S), 3TC(H), EFV(H)
IR.KB12RT	Male	41	18-11-2006	IVDU	B	TDF(S), 3TC(S), EFV(S)
IR.KB13RT	Male	40	Unknown	IVDU	A	TDF(S), 3TC(L), EFV(S)
IR.KB14RT	Male	38	07-08-2006	Hemophiliacs	A	3TC(S), TDF(S), EFV(S)
IR.KB15RT	Male	44	27-04-1999	Hemophiliacs	A	3TC(S), TDF(S), EFV(S)
IR.KB16RT	Female	33	04-07-2006	Homosexual	A	3TC(S), TDF(S), EFV(S)
IR.KB17RT	Male	35	30-10-2005	IVDU	A	TDF(S), 3TC(S), EFV(H)
IR.KB18RT	Female	38	18-03-2006	Homosexual	A	TDF(S), 3TC(S), EFV(S)
IR.KB19RT	Male	38	Unknown	Hemophiliacs	B	AZT(S), 3TC(S), EFV(S)
IR.KB20RT	Male	41	17-06-2006	IVDU	A	AZT(S), 3TC(S), EFV(S)
IR.KB21RT	Male	51	12-09-2006	IVDU	B	AZT(S), 3TC(S), EFV(S)
IR.KB22RT	Male	35	Unknown	IVDU	A	AZT(S), 3TC(S), EFV(S)
IR.KB23RT	Male	30	20-02-2008	IVDU	B	AZT(S), 3TC(S), EFV(S)
IR.KB24RT	Female	27	03-03-2008	Homosexual	B	TDF(L), 3TC(H), EFV(H)

<sup>1</sup>IVDU: Intravenous drug users, <sup>2</sup>Resistance analyzed using the Stanford HIV Resistance Database based on the entire sequence. (S) Stands for Susceptible, (L) Stands for low level resistance, (P) Stands for Potential low level resistance, (I) Stands for intermediate resistance and (R) stands for highly resistant.



**Figure1.** Un-rooted phylogenetic tree constructed using kimura two parameter matrixes and Neighbor-Joining method, based on reverse transcriptase sequence with MEGA4 software. Reference sequence from HIV genotypes are identified by gene bank accession number. Iranian sequences indicated by IR.KB...RT.

patients. The detailed study of the sequences generally confirmed the results obtained in previous studies which reported subtype A viruses were dominant among Iranian injection drug users (Soheilli *et al.*, 2009; Tagliamonte *et al.*, 2007; Naderi *et al.*, 2006; Sarrami-Forooshani *et al.*, 2006).

Of the 24 patients, 23 (95%) had resistance to NRTIs, 8 individuals (32%) to NNRTIs and one patient was susceptible to NRTIs and NNRTIs. The

distribution of resistance-related mutations in reverse transcriptase sequences was as follows: E44A (91%), K65R (4%), D67N (4%), V75A (95%), M184V/L (30%), T215Y/V (8%), and K103N (22%), Y181C (4%). The mutations indicative for resistance found in the study population are listed in Table 3. In the reverse transcriptase gene, NRTI-resistant mutation M184V appeared more prevalent in subtype A1 rather than subtype B and for NNRTI-resistant mutation

**Table 3.** Number and percentage of mutations revealed by Stanford HIV Resistance Database.

Percentage	Number	NNRTI	Percentage	Number	NRTI
4.3	1	A98G	4.3	1	M41L
4.3	1	L100I	91.3	21	E44A
21.7	5	K103N	4.3	1	K65R
4.3	1	Y181C	95.6	1	D67N
4.3	1	Y188L	95.6	22	T69I
			4.3	1	L74A
			95.6	22	V75A
			4.3	1	F77C
			4.3	1	Y115F
			8.6	2	V118IA
			8.6	2	Q151P/L
			30.3	7	M184V/L
			4.3	1	L210W
			8.6	2	T215Y/V
			8.6	2	K219E/Q

K103N also appeared more common with subtype A1.

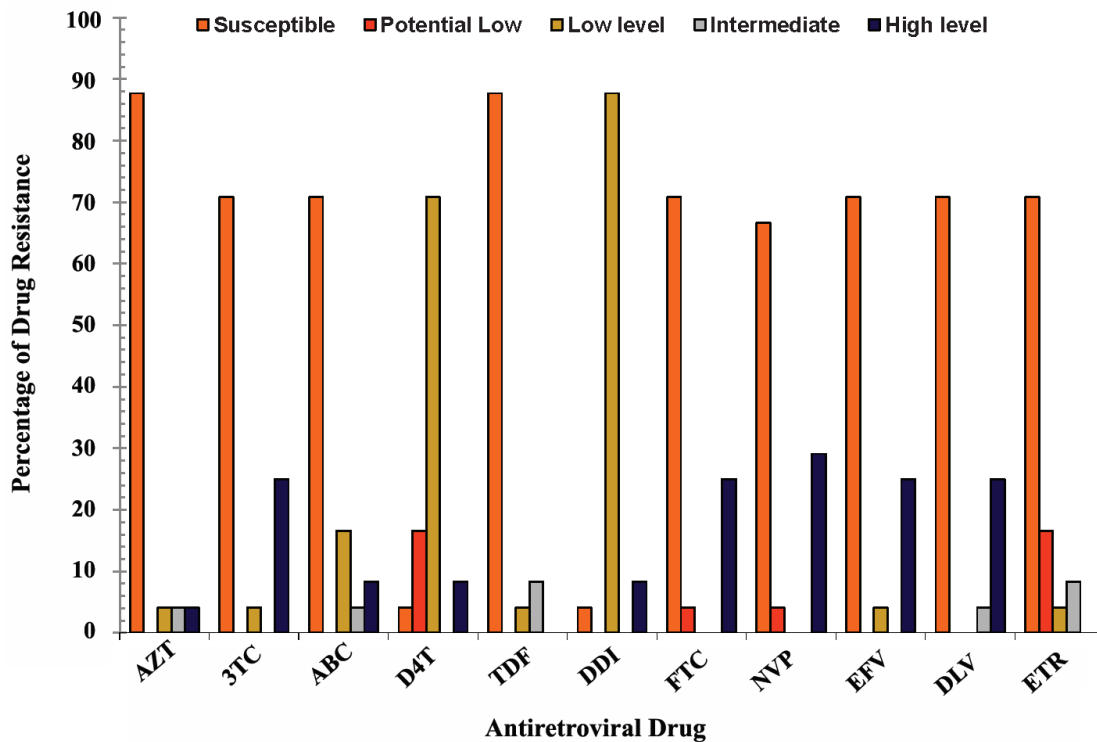
The drug resistance interpretation in this study showed: 87.7% susceptible, 4.1% low-level resistance, 4.1% intermediate and 4.1% high-level resistance for AZT, 70.8% susceptible, 4.1% low-level resistance and 25% high-level resistance for 3TC, 87.7% suscep-

tible, 4.1% low- level resistance and 8.3% intermediate for TDF, 66.6% susceptible, 4.1% low-level resistance and 29.1% high-level resistance for NVP and 70.8% susceptible, 4.1% low-level resistance and 25% high-level resistance for EFV. The results are summarized in Figure 2.

## DISCUSSION

Current data indicate that, 23 of 24 patients (95.8%) had RT mutations associated with reduced susceptibility to the NRTIs from low to high level and 8 of 24 patients (32%) had RT mutations associated with reduced susceptibility to the NNRTIs.

In one sample (IR.KB2RT) with drug regimen include 3TC, TDF and EFV (past drug regimen: AZT, 3TC and NVP) there was M41L, L210W, T215Y, K219E (thymidine Analog Mutations) and M184V mutation to the NRTIs and K103N mutation to the NNRTIs that these mutations cause high level resistance to AZT, 3TC, ABC, D4T, NVP and EFV and intermediate resistance to TDF. In IR.KB4RT (M184V), IR.KB5RT (M184V and Y181C), IR.KB7RT (M184V and K103N), IR.KB3RT (M184V and K103N), IR.KB11RT (M184V and K103N),



**Figure 2.** Percentage of antiretroviral drug resistance among Iranian HIV infected patients.



IR.KB17RT (K103N) and IR.KB24RT (K65R, M184V and Y188L) patients there is high level resistance to 3TC in all patients mentioned above except IR.KB17RT, as well as high level resistance to NVP and EFV in all patients mentioned above except IR.KB4RT and high level resistance to ABC and D4T and intermediate resistance to AZT and TDF in IR.KB24RT patient that had sexual contact with transmitted drug resistance patient probably as a risk factor. Thymidine analogs mutation (TAM) cause higher levels of phenotypic and clinical resistance to the thymidine analogs (AZT, D4T) and cross-resistance to ABC and TDF. Three TAM markedly reduces the clinical response to ABC and TDF (Eron *et al.*, 2006; Gallant *et al.*, 2006; Shafer *et al.*, 2003).

M184V is the most commonly occurring NRTI resistance mutation. *In vitro*, it causes high-level resistance to 3TC and Emtricitabine (FTC), low-level resistance to ABC and increased susceptibility to ZDV, d4T, and TDF (Campbell *et al.*, 2005; Nijhuis *et al.*, 1997; Larder *et al.*, 1995). The frequency of M184V mutation in this study was 30.3% (Table 3). Data from multiple 3TC-containing dual-NRTI regimens also suggest that 3TC continues to exert an antiviral effect even in patients whose virus isolates contain M184V (Shafer and Schapiro 2008). The presence of K65R in combination with M184V is sufficient for high-level resistance to both ABC and D4T (Shafer and Schapiro, 2008; Doualla-Bell *et al.*, 2006; Kyriakides, 2000).

The frequency of K103N mutation in this study was 21.7% (Table 3) that is currently the most clinically important NNRTI resistance mutation because it causes 20 to 50- fold resistance to each of the available NNRTIs (Marconi *et al.*, 2007; Shafer *et al.*, 2000). The frequency of mutations that confer resistance to NRTI (95%) and NNRTI (32%) in the current study were higher and lower than that found in other study in Iran respectively (Hamkar *et al.*, 2010). Other differences include existing of A98G mutation and existing of drug resistances in subtype A in this study but these are not in Hamkar study. These differences are probably due to difference in the type of medication regimen. Majority of patients in this study were using the 3TC plus TDF plus EFV regimen and in the Hamkar study nearly all patients were using the 3TC plus AZT plus NVP regimen.

HAART regimen therapy consists of the 2 NRTI plus 1 NNRTI or 1 PI. In Gupta study (2008), the analysis of clinical trials involved nearly 8000 patients, the prevalence of M184V and K65R mutations at the time of virologic failure (Inability of anti-

HIV drug treatment to reduce viral load or to maintain suppression of viral load) is significantly higher in patients using first-line NNRTI versus PI-based HAART. The results of this study suggests that the genotypic resistance is detected more frequently after failure of NNRTI-based HAART than after failure of PI-based HAART (Ravindra *et al.*, 2008). However, our data suggest that probably, the use of 2 NRTIs plus 1 PI regimen is better than 2 NRTIs plus 1 NNRTI regimen in Iranian patients that use 2 NRTI plus NNRTI regimen because PI inhibitors have high genetic barrier than NNRTI inhibitors.

Most samples were obtained from drug abusers clustered with a common node close to reference subtype A1. The subtype B Iranian isolates did not cluster in a tight group with subtype B, exception IR.KB28TR, IR.KB191RT and IR.KB3525RT that they had the highest homogeneity with subtype B. Our data support the need for continuous surveillance of resistance patterns to help guide therapeutic approaches and limit transmission of these variants in Iranian patients.

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