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ORIGINAL ARTICLE

Antiretroviral drug resistance mutations in naïve and experienced patients in Shiraz, Iran, 2014

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Abstract Resistance to antiretroviral agents is a significant concern in the clinical management of HIV-infected individuals, particularly in areas of the world where treatment options are limited. In this study, we aimed to identify HIV drug-resistance-associated mutations in 40 drug-naïve patients and 62 patients under antiretroviral therapy (ART) referred to the Shiraz HIV/AIDS Research Center – the first such data available for the south of Iran. HIV reverse transcriptase and protease genes were amplified and sequenced to determine subtypes and antiretroviral- resistance-associated mutations (RAMs). Subtype CRF35-AD recombinant was the most prevalent in all patients (98 of 102, 96 %), followed by subtype A1, and subtype B (one each, 2 %). Among the 40 ART-naïve patients, two mutations associated with nucleoside reverse transcriptase inhibitor (NRTI) resistance (two with Y115F and T215I) and three associated with non-nucleoside reverse transcriptase inhibitor (NNRTI) resistance (two with G190S and Y181C, four with V179T) were found. Among ART-experienced patients, four mutations associated with resistance to NRTI, four with NNRTI, and five with protease inhibitors (PI) were found. Twenty patients with high levels of resistance were already on second-line therapy. We document for the first time in this region of Iran high levels of ART resistance to multiple drugs. Our findings call for more vigilant systematic ART resistance surveillance, increased resistance testing, careful management of patients with existing regimens, and strong advocacy for expansion of available drugs in Iran.

Introduction

Although not curative, combinational antiretroviral therapy (ART) has proven to be effective in controlling HIV replication. Currently, there are six different classes of regimens to treat HIV, the three most common in Iran being nucleoside and nucleotide analogue reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), and protease inhibitors (PI) [1]. ART generally includes two NRTI and one NNRTI. For HIV-positive patients in Shiraz, zidovudine (AZT), lamivudine (3TC) and efavirenz (EFV) are used as the first-line therapy. When treatment fails, second-line therapy usually includes 3TC, tenofovir (TDF), and Kaletra (lopinavir/ritonavir).

Regardless of the significant achievements in terms of viral suppression, resistance to ART agents is a concern, as it can lead to treatment failure [2]. ART resistance results from mutations appearing across the genome, targeting viral proteins [1]. As many as 50 % of patients in the US show resistance to at least one available antiretroviral drug

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[3]. The US, however, has multiple drug classes and agents to use when treatment fails. In much of the world, few options are present beyond a first and second line. Fewer still are currently available for wide use.

HIV genotype diversity is a result of high mutation rates of the reverse transcriptase gene combined with a high viral turnover rate under the selective pressure of ART and the immune system [4]. Some studies link differences between subtypes to the rate of HIV disease progression [5], while other studies have not found such differences [6]. HIV subtypes might be important for the development of resistance to ART agents. There are also studies on the effect of subtype on the outcome of ART in patients, with some reporting differences between B and non-B subtype viruses in the development of drug resistance [7]. Other studies report no differences on the outcome of treatment by subtype [8].

Primary drug resistance is a major concern, as an ART-naïve patient may carry resistant virus without any prior treatment [9]. Indeed, 7-17 % of naïve patients have ART resistance in high-income countries in North America and Western Europe, whereas in middle- and low-income countries, an estimated 7 % have ART resistance [10]. The prevalence of primary resistance in counseling and behavioral centers in Tehran and nine provinces of Iran has been reported to be 5-15 % [11–13], with CRF35_AD as the predominant subtype [12, 14]. However, no data on primary or secondary ART resistance have been reported from the south of Iran. We therefore present the results of the first study to determine ART-resistance-associated mutations among newly diagnosed ART-naïve and ART-experienced patients in Shiraz, southern Iran's most populous city.

Materials and methods

Study participants

For the purpose of this cross-sectional study, 108 HIV-positive patients consecutively seen at the Counseling Behavior Modification Center in Shiraz from April 2013 to February 2014 were recruited. Of these, 40 were newly diagnosed ART-naïve, and 68 had been under treatment for at least one year, using a regimen that included two NRTIs with one NNRTI (NNRTI-based regimen) for 48, and two NRTI with combination of PI (PI-based regimen) for the remaining 20. Informed consent was obtained from each patient.

The study protocol was approved by the Committee of Medical Research Ethics in Golestan and Shiraz University of Medical Sciences.

RNA isolation, amplification, and genotyping

Blood samples were obtained in sterile EDTA-containing tubes, and plasma was separated and stored at -70 °C. RNA was extracted from plasma according to the manufacturer's protocol (Roche Total RNA and DNA Extraction Kit). cDNA synthesis was performed at 50 °C for 30 minutes using a one-step kit as indicated by the manufacturer (Invitrogen).

Two parts of the HIV *pol* gene were amplified by nested RT-PCR following standard procedures. Briefly, the HIV protease gene was amplified using the following reaction mixture: 5 μL of cDNA, 4.5 μL of 10X PCR buffer, 1 μL of 10 mM dNTP, 0.3 μL of 5 U pfu polymerase, 6 μL of 100 mM $MgCl_2$, 0.1 μL of each PCR primer (10 pM) and 37 μL of DEPC-treated water. The final amplified region covered the HIV protease gene from codon 9 to codon 99. The following primer pairs were used: outer sense primer (SEQ ID NO -1), 5' CAG AGC CAA CAG CCC CAC CAG3'; outer antisense primer (SEQ ID NO -2), 5' ATC AGG ATG GAG TTC ATA ACC CAT CCA 3'; nested sense primer (SEQ ID NO -3), 5' CCT CAR ATC ACT CTT TGG CAA CG 3'; nested antisense primer (SEQ ID NO -4), 5' CTG GTA CAG TYT CAA TAG GRC TAA T [15]. Annealing was done at 57 °C, extension at 72 °C and denaturation at 94 °C. Each step of a cycle was carried out for 1 minute. The outer PCR consisted of 40 cycles, and the nested round consisted of 35 cycles. A final extension step at 72 °C was carried out for 2 minutes.

The RT gene was amplified using the following reaction mixture: 5 μL of cDNA, 4.5 μL of 10X PCR buffer, 0.25 μL of 40 mM dNTP, 0.3 μL of 5 U pfu polymerase, 3 μL of 100 mM $MgCl_2$, 0.1 μL of each PCR primer at 10 pM and 39 μL of DEPC-treated water. The amplicon was a 665-bp region encoding amino acids 17 to 237 of the RT gene, and the following primer pairs were used: outer sense primer (RT-1), 5' GTT GAC TCA GAT TGG TTG CAC 3'; outer antisense primer (RT -2), 5' GTA TGT CAT TGA CAG TCC AGC 3'; nested sense primer (RT -4), 5' GGA TGG CCC AAA AGT TAA AC 3'; nested antisense primer (RT -3), 5' TAT CAG GAT GGA GTT CAT AAC [16]. The PCR conditions were as follows: 94 °C for 5 min followed by 35 cycles of 94 °C for 30 seconds (denaturation), 55 °C for 30 seconds (annealing), and 72 °C for 1 minute (extension). A final extension step at 72 °C was carried out for 4 minutes.

Both PCR products were visualized on a 2 % agarose gel with ethidium bromide staining. The PCR products were decontaminated using a gel purification kit (Bioneer) and sequenced on both strands in an automated DNA sequencer (ABI PRISM 3730 version 3.0, Applied Biosystems, Foster City, CA).

Subtype and drug resistance interpretation and phylogenetic tree

Sequences were corrected using Bioedit software (ver. 7.0.5.3). The protease and RT sequences were analyzed using the Stanford University HIV Drug Resistance mutations databases (version 4.2.6 [http://hivdb.stanford.edu]) for determination of drug resistance in patients [17].

Phylogenetic analysis was done by the neighbor-joining method with 1000 bootstrap replicates and Kimura's two-parameter correction. The phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) software, version 4 [18].

The pol nucleotide sequences reported in this study have been deposited in the GenBank database under accession numbers KX086583-KX086683.

Results

A total of 108 HIV-positive patients were initially included in this study, for whom 102 samples were suitable for sequencing. The characteristics of these 102 participants are shown in Table 1. Of the 40 ART-naïve patients, 35 % were female, the mean age was 37 years (SD ±7), and the mean CD4 count was 391 (SD ±163). Reported transmission routes were 45 % intravenous drug use, 30 % had an HIV-infected husband, 15 % had multiple sex partners, 5 % were reported to have been infected through blood products, and 5 % had no risk reported. Among the 62 ART-experienced patients, 42 % were female, the mean age was 41 years (SD ±6), and the mean CD4 count was 202 (SD ±107). Reported transmission routes were 48 % intravenous drug users, 26 % HIV-infected husband, 16 % multiple sex partners, 7 % blood products, and 3 % tattooing.

Most patients (48, 77 %) on ART used combined doses of AZT, 3TC, and EFV as the first-line therapy for at least one year. The remainder were using 3TC, TDF, and Kaletra as second-line therapy.

Subtype CRF35-AD recombinant was the most prevalent in all patients (98 of 102 or 96 %), followed by subtype A1, and subtype B (each 2 %).

Several mutations associated with NRTI and NNRTI resistance were detected in ART-naïve patients (Table 2). Detected ART-resistance-associated mutations related to NRTI were Y115F (two, or 5 % of the patients) and T215I (5 % of the patients). Y115F reduces abacavir (ABC) susceptibility ~3-fold but has little phenotypic effect on tenofovir (TDF), and T215I does not decrease NRTI susceptibility but arises from patients primarily infected with strains containing T215Y/F. Y181I (two, or 5 % of the patients), G190S (5 % of the patients), and V179T (four, 10 % of the patients) were mutations related to NNRTI. By patients, accounting for multiple mutations in individuals, NRTI resistance was present in 5 %, and NNRTI in 10 %. No major PI drug RAMs were seen among ART-naïve individuals. In the ART-naïve patients, only two (5 % of

Table 1 Characteristics of HIV-positive patients according to antiretroviral treatment (ART) status, Shiraz, Iran, 2014 (N = 102)

Characteristic	ART-naïve N (%) or mean (SD)	ART-experienced N (%) or mean (SD)
Total	40 (100)	62 (100)
Sex:		
Male	26 (65)	36 (58)
Female	14 (35)	26 (42)
Mean age (years)	37 ± 7	41 ± 6
Mean CD4 count	391 ± 163	202 ± 107
Reported transmission route:		
Intravenous drug use	18 (45)	30 (48)
HIV-infected husband	12 (30)	16 (26)
Multiple sex partners	6 (15)	10 (16)
Blood products	2 (5)	4 (7)
Tattooing	-	2 (3)
Unreported/unknown	2(5)	-
Years of diagnosis	2011-2013	2007-2012
Years since start of treatment		
1	NA	32 (52)
2		10 (16)
3-5		16 (25)
5+		4 (7)

Table 2 Antiretroviral drug RAMs detected in HIV-positive patients in Shiraz, Iran in 2014 according to treatment history

Mutations detected in ART-naïve patients (n = 40)			Mutations detected in ART-experienced patients (n = 62)		
NRTI	NNRTI	PI	NRTI	NNRTI	PI
2 (Y115F)	2 (G190S)	-	6 (V75M)	6 (K103N)	6 (M46L/I)
2 (T215I)	2 (Y181I)	-	6 (M184V/I)	2 (P225H)	2 (V82C)
-	4 (V179T)	-	2 (K65N)	2 (V179T)	2 (V82A)
-	-	-	2 (K219Q)	2 (Y181C)	2 (I54V)
-	-	-	-	-	2 (L90M)

NRTI, nucleoside reverse transcriptase inhibitor; NNRT, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor

Table 3 Levels of antiretroviral drug resistance in HIV-positive patients in Shiraz, Iran in 2014 (N = 102) according to treatment history

ART-experienced patients (n = 62)							ART-naïve patients (n = 40)		
	First-line therapy (n = 42) (2NRTI+1NNRTI)			Second-line therapy (n = 20) (2NRTI+1PI)			No therapy		
	Level of resistance			Level of resistance			Level of resistance		
NRTI	High n (%)	Intermediate n (%)	Low n (%)	High n (%)	Intermediate n (%)	Low n (%)	High n (%)	Intermediate n (%)	Low n (%)
3TC	-	-	2 (5)	6 (30)	-	-	-	-	-
ABC	-	-	2 (5)	-	-	6 (30)	-	2 (5)	-
AZT	-	-	-	-	-	2 (10)	-	-	2 (5)
D4T	-	-	-	4 (20)	-	2 (10)	-	-	2 (5)
DDI	2 (5)	-	2 (5)	-	4 (20)	6 (30)	-	-	2 (5)
FTC	2 (5)	2 (5)	-	6 (30)	-	2 (10)	-	-	-
TDF	-	-	2 (5)	-	-	-	-	-	2 (5)
NNRTI									
EFV	4 (10)	2 (5)	-	2 (10)	-	-	2 (5)	2 (5)	-
ETR	-	-	-	-	2 (10)	-	2 (5)	-	2 (5)
NVP	4 (10)	-	2 (5)	2 (10)	-	-	4 (10)	-	-
PRV	-	-	-	-	2 (10)	-	2 (5)	-	2 (5)
PI									
ATV	-	-	2 (5)	-	4 (20)	4 (20)	-	-	-
DRV	-	-	-	-	-	-	-	-	-
FPV	-	-	2 (5)	-	4 (20)	4 (20)	-	-	-
IDV	-	-	2 (5)	-	4 (20)	4 (20)	-	-	-
LPV	-	-	2 (5)	-	2 (10)	6 (30)	-	-	-
NFV	-	-	2 (5)	2 (10)	2 (10)	4 (20)	-	-	-
SQV	-	-	2 (5)	-	4 (20)	-	-	-	-
TPV	-	-	2 (5)	-	-	2 (10)	-	-	-

NRTI, nucleoside reverse transcriptase inhibitor; NNRT, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor

the patients) individuals had multiple mutations, namely Y181I, Y115F, and T215I.

The genotyping resistance interpretation algorithm indicated resistance to AZT (two, or 5 % of the patients), D4T (5 % of the patients), DDI (5 % of the patients) for NRTI, and resistance to EFV (four, or 10 % of the patients), NVP (10 % of the patients), ETR (10 % of the patients), and PRV (10 % of the patients) for NNRTI (Table 3).

In the ART-experienced patients, major mutations associated with resistance to NNRTI were K103N (six, or 10 % of the patients), P225H (two, or 3 % of the patients),

V179T (3 % of the patients) and Y181C (3 % of the patients) (Table 2). Detected NRTI mutations were V75M and M184I/V (six, or 10 % patients each), K219Q and K65N (two each, or 3 % of the patients). Mutations associated with PI among ART-experienced patients were M46L/I (six, or 10 % of the patients), and I54V, L90M, V82C, and V82A (two each, or 3 % of the patients). All patients with PI mutations had PI in their current or past regimens. In addition, minor PI RAMs were L10I/V (eight, or 13 % of the patients) and A71T (four, or 6 % of the patients). Among the 62 ART-experienced patients, 26 (42 % of the patients) had at least one drug RAM, twenty (32 % of the patients) had RAMs to one drug class, six (10 % of the patients) had resistance to two drug classes, and two (3 % of the patients) had RAMs to all three drug classes assessed. In these patients, resistance to NRTI, NNRTI, and PI were 26 %, 13 %, and 16 %, respectively. The genotyping resistance interpretation algorithm indicated 23 % resistance to DDI, 10 % resistance to D4T, and 10 % resistance to 3TC in the NRTI group and 13 % resistance was seen to EFV and NVP. Of the ART-experienced patients, ten (16 % of the patients) had multiple mutations.

In addition to RAMs, there were other polymorphisms in our sequences that had no association with drug resistance. For example, V179I, V60I, K122E, L234P, H235P in the RT region and L89M, E35D, M36I, and H69K in the PR region are common polymorphisms in our study that are not associated with decreased NNRTI and PI susceptibility, respectively.

Discussion

Tracking primary and secondary ART resistance provides important patient-care and public-health information, which is particularly critical in settings with few treatment options and lines of therapy. We present the first study of ART resistance in the south of Iran, in Shiraz, and found substantial transmitted resistance and resistance among patients on first- and second-line treatment. Levels reported in this region are comparable to elsewhere in Iran [11, 19]. We also confirm that the most common subtype was subtype CRF35-AD as elsewhere in the country [12, 14, 20–22]. Any resistance is a potential threat because inadequate suppression of HIV replication by ART affects individual patient care as well as onward transmission of infection.

Transmission of resistant viruses is a particular concern when considering initiation of ART in new patients. In ART-naïve patients, we found 10 % resistance to NNRTI and 5 % to NRTI, a finding consistent with previous studies in Iran that have calculated primary resistance at 5 to 15 % [11, 13, 23]. When the frequency of transmitted drug resistance is estimated at 8 %–10 %, drug-resistance testing before commencement of ART in untreated patients is cost-effective [24]. In the ART-naïve patients, we found two NRTI RAMs and three NNRTI RAMs. Mutation V179T was reported in four individuals and this might contribute to reduced ETR susceptibility in combination with other mutations. Mutations Y181I and G190S were similar to the profile of mutations found by Smith *et al.* [25]. Our rate of primary resistance in ART-naïve participants was lower than among patients in the United States, Europe, Australia, and Japan, where it was estimated to be between 10 % and 17 % by WHO [26]. Fortunately, for the present, we detected no major PI drug RAMs among ART-naïve patients. This phenomenon is probably caused by the higher genetic barrier for PI resistance and not having PI in the first-line regimen in Iran. PI-associated RAMs in our ART-experienced patients was 16 %, similar to other Iranian studies [11, 19].

Our data allowed us to identify mutations associated with ART resistance among those undergoing both first- and second-line treatment. The K103N mutation was the most common NNRTI mutation detected in ART-experienced patients. Recently, K103N was shown to be the most important NNRTI RAM due to the 20- to 50-fold increase in resistance against each of the available NNRTIs [27]. The proportion of patients with a NNRTI-resistance-associated mutations is higher in treated patients than in drug-naïve patients, probably due to poor ART adherence and pharmacologic pressure [28]. We found highest resistance for EFV and NVP, both of which are used in first-line therapy. Resistance to 3TC, 13 % here, is lower than in previous studies that reported 53 %, 60 % and 25 % in Iran [11, 19, 28]. Use of ART increases the chance of resistance in patients with incomplete viral suppression, and it may cause treatment failure [29]. The highest resistance rate (26 %) in treatment-experienced participants was seen against NRTI, which is similar to the results reported by Hamkar et al. [19]. The most prevalent mutation was V75M, which is related to resistance to stavudine (D4T) and didanosine (DDI). Another common mutation related to NRTI was M184I/V in six patients. This mutation can cause high-level resistance to 3TC and emtricitabine (FTC) and low-level resistance to abacavir (ABC) and DDI. A mutation in position 184 was the most prevalent mutation associated with resistance to NRTI in other studies [23]. The high level of resistance to 3TC is the result of the M184V/I mutation. When only 3TC is used as a drug regimen, resistant strains become the main strain in a few weeks [30], and M184V is the first mutation to appear when 3TC is used in an ART regimen [1]. Among patients receiving ART, resistance to 3TC was more common for PI-based regimens, probably as a result of second-line

therapy in these patients. More investigation is needed to confirm this. Although FTC use was not reported, accumulation of NRTI mutations in patients with PI-based regimens on 3TC resulted in frequent high-level (30 %) or low-level (10 %) cross-resistance to FTC.

Among twenty patients on second-line therapy, two patients had a combination of RAMs to all three drug classes (NRTI, K219Q; NNRTI, K103N and Y181C; PI, I54V and V82A), probably as a result of non-adherent to drugs in these patients. On the other hand, there were six cases with combination of RAMs to NRTI and NNRTI.

The profile of mutations in the protease region was different from those in other Iranian studies. For example, RAMs to PI such as A71T, L10V, L10F, Q58F, I50V, and I84V have been reported by Hamkar et al. and Baesi et al. [11, 19]. PI RAMs were seen in 16 % of patients, and as expected, they were less frequent than reverse transcriptase inhibitor mutations because of a natural genetic protease barrier to mutation [28]. Although patients on first-line therapy did not receive PI, low-level resistance associated with PI mutations was detected in this group. We think this might be due to drug-selective pressure or transmitted drug resistance. Consistent with a previous report, maximum and minimum resistance was reported against nelfinavir (NFV), saquinavir (SQV), and darunavir (DRV) [11].

We recognize the limitations of our study. First, the overall sample size is small. Second, the study population was recruited from those visiting clinics. We do not have information on the level of primary and secondary resistance in the community at large.

Conclusion

In much of the world, genotyping is widely used in clinical settings, with evidence from several trials supporting their efficacy in improving patient management [31]. Most of the time in Iran, treatment is started without genotyping testing. With due attention to the presence of primary resistance in drug-naïve patients as shown in our study, the importance of genotyping assay for better clinical outcome and inhibition of resistance in these patients should be evident.

Our first study on drug resistance among HIV-positive patients in Shiraz emphasizes the need for resistance testing before initiation of therapy in drug-naïve patients in order to select the best therapeutic approaches, and before switching to second-line therapy for other patients. In addition, we recommend a more systematic surveillance for resistance patterns and further studies to assess major mutations associated with antiretroviral drug resistance in Iranian patients. Finally, given the current situation and the likelihood of developing and transmitting resistance, there needs to be advocacy for increased availability and affordability of newer ART options.

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Compliance with ethical standards

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