

Quantitative Transmitted Drug Resistance (TDR) Variation in Acute/Recently Infected Men who have Sex with Men (MSM) Chinese HIV Patient Cohort

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Abstract

Transmitted drug resistance (TDR) is an ongoing public health problem in HIV disease treatment and increases the risk of virologic failure following combination anti-retroviral therapy (cART) initiation. Current HIV treatment guidelines recommend resistance testing at entry into HIV care and at cART initiation. In this study, we longitudinally observed 10 acute/recently infected patients for 24-51 months. In addition to calculating the replacement rate of TDR measured by bulk population sequencing, High-throughput sequencing (HTS) was used to detect the minority mutations and quantify the variation of each TDR mutations during the follow-up. We compared the predictions of virus sensitivity to each drug by bulk population sequencing alone or by the combined sequencing with bulk population sequencing and HTS, which was analyzed with their virologic response after 6 months antiretroviral therapy. Our observations provided insights into the significance of low-frequency HIV-1 drug resistance mutations in acute/recently infected patients, and whether or not TDR are likely to revert, or persist as minority species or be stable. This study not only provides valuable data on TDR prevalence but also has important implications for the clinical management of these patients.

Keywords: Transmitted drug resistance (TDR); Anti-retroviral therapy; High-throughput sequencing (HTS); Mutations Virologic response

Introduction

Drug-resistant mutants can be transmitted to the patients who haven't been exposed to any antiretroviral drugs, which are called transmitted drug resistance (TDR). TDR is an ongoing public health problem all over the world, may increase the risk of virologic failure following combination anti-retroviral therapy (cART) initiation [1]. Therefore, current HIV treatment guidelines recommend resistance testing at entry into HIV care and at cART initiation. However, the stability of these mutants after the transmission to a new host in the absence of selective drug pressure is not known. Several studies believed that, in contrast to acquired drug resistance which will decline rapidly after the drug interruption, transmitted drug resistance mutations (DRMs) are usually not associated with a reduced viral replication capacity, and because of the archived mutation(s) in latently infected resting CD4+ T cells, the TDR mutations reversed to wild-type HIV (back mutation) after the transmission [2-5]. Nonetheless, reversion of the transmitted drug resistance patterns in the plasma has been reported as well [6,7]. Furthermore, some studies reported a higher level of resistance in recently infected individuals compared with individuals with a longer or unknown duration of infection, suggesting that reversion of resistant viruses to wild-type variants does occur rather frequently over time [8,9]. Recent advances in high-throughput sequencing (HTS) have revolutionized HIV-1 sequencing and make it possible to study the HIV-1 drug-resistant viruses at levels below 20%-30%, which is called low-frequency HIV-1 drug resistance mutations or minority variants. Earlier data indicated that secondary resistance can be detected in the blood plasma as minority variants up to 12-24 months before returning to basal background levels [10], which can still have significant clinical implications on the risk of combination antiretroviral treatment (cART) failure [11]. However, to our knowledge, very few studies have been done to observe quantitative TDR variation in acute/

recently infected patients. When sexual transmission of HIV occurs, it is believed that selection of highly fit drug-resistant variants occurs and which persist for years. The prolonged persistence of transmitted drug resistance strongly supports the routine therapeutic use of HIV resistance genotyping for all newly diagnosed individuals. TDR that goes undetected can jeopardize response to first-line therapy if that regimen includes drugs compromised by resistant virus; therefore evaluation of HIV TDR has great significance for both HIV prevalence and HIV therapy. Drug-resistant strains could reach high frequencies among newly infected patients, which would render certain routinely used antiretroviral drugs useless; however this has never happened with HIV drugs. Transmitted drug resistance does occur in HIV, but the numbers have remained relatively low. Comparative data for Malaria shows that many malaria drugs have been withdrawn from use by world health authorities because of widespread transmission of drug resistant malaria parasites.

Limited information is available about the prevalence and variation of pre-existing minority drug-resistant in acute/recently infected patients China. In this study, we longitudinally observed 10 acute/recently infected patients for 24-51 months. In addition to calculating the replacement rate of TDR measured by bulk population

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sequencing, HTS (Illumina) was used to detect the minority mutations and quantify the variation of each TDR mutations during the follow-up. We compared the predictions of virus sensitivity to each drug by bulk population sequencing alone or by the combined sequencing with bulk population sequencing and HTS, which was analyzed with their virologic response after 6 months antiretroviral therapy. Insights into the significance of low-frequency HIV-1 drug resistance mutations in acute/recently infected patients, and whether or not TDR are likely to revert, or persist as minority species or be stable has important implications for prevalence studies, clinical management of these patient and over all public health.

Materials and Methods

Study population

Our study comprised 186 acute/early HIV infected patients belonging to the men who have sex with men (MSM) Cohorts, and who do not have any antiretroviral drug exposure and were enrolled in the AIDS High Risk Cohort Program clinics at the Youan hospital, Beijing, China, between 2007 and 2010. This program is supported by the Beijing Science & Technology Committee. All the participants in this Cohort were assessed for HIV-1 antibodies (enzyme-linked immunosorbent assay, EIA) and HIV-1 RNA (measured by the real-time RT-PCR COBASTM Ampliprep/COBASTM Taqman HIV test; Roche Diagnostics; sensitivity at 20 copies/mL) every two months [12,13] until HIV-1 antibodies or HIV-1 RNA turns to be positive. Acute/early HIV-1 infection was defined as (1) positive HIV-1 RNA with a negative or indeterminate HIV-1 antibody test, followed by HIV sero conversion within 6 months; (2) a positive HIV-1 antibody test, with a history of a negative HIV-1 antibody test within the previous 6 months. We enrolled participants meeting the following 3 criteria in the study: (1) baseline genotyping (at the date of diagnosis) was performed; (2) >24 months of observed follow-up time without ART; (3) plasma samples were collected at 6 months, 12 months and 24 months after the estimated time of infection (ETI) during the follow up for genotype testing purpose. ETI was estimated as follows: (1) Mid-point between the last sero-negative date and the first sero-positive date; (2) 14 days prior to the date of HIV RNA turned to positive; (3) date of positive HIV antibody testing (EIA) and indeterminate western blot outcomes minus 28 days.

Clinical and laboratory evaluations

Demographic and behavioral data were collected by trained counselors using a standardized questionnaire at enrolment on HIV diagnosis date. CD4 count and HIV-1 viral load were measured at baseline and every 6 months.

HIV-1 subtype classification and recombination

Neighbor-joining phylogenetic trees with maximum composite likelihood substitution model were constructed by Mega v 5.03 software to determine HIV-1 subtype.

HIV genotype determinations and drug sensitivity prediction

HIV genotype testing was performed using bulk population sequencing and high-throughput sequencing (Illumina) respectively. TDR was defined as the detection of 1 or more mutations in the surveillance drug resistance mutations (SDRMs) listed by the World Health Organization [14]. Drug sensitivity was calculated using code developed by Frontier Science and scores from the Stanford HIV DBAlgorithm, version 6.2.0 [15].

Population-based sequencing

Population-based sequencing which is a much less sensitive method (detection limit for drug-resistant HIV-1 is approximately 20%) was performed using a published in-house method [13] that targets 1.3 kb region of the pol gene, covering the complete protease (amino acids 1-99); the part of the reverse transcriptase (amino acids 1-305) nucleotide. Mutations were ascertained by a comparison of aligned sequences with the Los Alamos National Laboratory subtype B consensus sequence.

High-Throughput Sequencing (HTS)

The same sample as population-based sequencing was used for high-throughput sequencing (HTS). HIV-1 RNA was extracted from 1 ml of plasma using QIA amp viral RNA extraction kits (Qiagen, Courtaboeuf, France). Reverse-transcription was performed with random primers and Superscript III RT (Invitrogen, Carlsbad, CA) followed by nested PCR, the PCR product was then purified (QIAquick Gel Extraction Kit). DNA sample was sequenced on the Illumina HiSeq 2000 according to the manufacturer's protocols to generate 100-bp paired-end reads.

The original data was analyzed FastQ format, using NGS QC Toolkit (v2.3) for quality control [16], trimming back reads with quality 20 from the end, reads shorter than 75-bp were discarded (perl Trimming Reads.pl -i data1.fq -q 20 -n 75 -o data1_filtered.fq). Reads from each isolate were mapped to reference sequence (RT and Protease) using Burrows-Wheeler Aligner (BWA) [17] with default parameters. SNPs were identified using SAM tools [18] (using SAM tools pileup and removing low-confidence site with consensus base quality of ≤ 20 , read depth of ≤ 10). The minority resistant viruses were defined as $\geq 1\%$, and $\leq 20\%$.

Replacement of baseline TDR detected by bulk population sequencing

All the patients who were TDR positive on baseline genotyping, follow-up genotypes would be performed at 6 months, 12 months, 24 months and at the end of follow up by bulk population sequencing. When a baseline TDR mutation was not detected subsequently, it will be considered to have been replaced.

Quantify the variation of TDR Mutations during the follow up in 5 acute/recent infected patients

HTS was used to quantify the variation of each resistance-associated mutation (RAMs) at baseline and the last available specimen in 5 acute/recent infected patients (including 3 patients who seemed to have lost their TDR during the follow up). The absolute number of each RAM copies per mL of plasma was calculated by multiplying the proportion of RAMs by the plasma virus load.

Assessment of discrepancies between population and HTS in 10 acute/recent infected patients

According to the results of baseline genotyping (population sequencing), 5 TDR positive patients and 5 TDR negative patients were selected randomly. HTS was used to detect low-frequency HIV-1 drug resistance mutations, assess the discrepancies between population sequencing and HTS. Viral susceptibility to each drug was classified as "Sensitive", "Potential low-level resistance", "Low-level resistance", "Intermediate resistance" or "High-level resistance" according to the Stanford HIVDB 5-point resistance scale. Only "Intermediate

resistance” and: High-level resistance”, that is ≥ 15 score, were considered to be resistant to a certain kind of drug. Virologic response was defined as virologic suppression (A confirmed HIV RNA level below the limit of assay detection), incomplete virologic response (Two consecutive plasma HIV RNA levels >200 copies/mL after 24 weeks on an ARV regimen), or virologic rebound (Confirmed HIV RNA >200 copies/mL after virologic suppression) according to the “Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents” on 2/2013 [19].

Ethics statement

Informed consent was obtained from all study participants for the collection of blood samples and subsequent analyses, and the study was approved by the institution’s ethical committee of You an Hospital.

Statistics

Prevalence of TDR was calculated as the number of patients with detectable SDRMs divided by the number of all patients with an available genotype; Confidence intervals (CI) for proportions were calculated using a 95% Wilson confidence interval for binomially distributed data. All statistical analyses were conducted in SPSS 14.0 software.

Results

Replacement of baseline TDR

Of the 186 patients with baseline genotyping performed, 16 were discovered to have baseline TDR mutations (8.6%) by population-based sequencing. 6 of them were excluded because short of follow up (less than 24 months), the other 10 patients were measured of the TDR at 6 months, 12 months, 24 months after the ETI and at the end of follow up (24-51 months after ETI, at a median of 24 months). Seven patients (70%) presented as CRF01-AE subtype and 3 patients (30%) presented as B subtype, with a median of age at 26 (19-46), and presented a median of 30 days (range, 17 to 50 days) after their EDI at diagnose (baseline), with a median baseline CD4 count of 558 cells/mm³ (130-807) and 4.90 log₁₀ RNA copies/ml (3.51-7.78). TDR was measured at 6 months, 12 months, 24 months and the end of follow up respectively by bulk population sequencing. All of the participants remained treatment naive for the duration of the study. All subjects reported sex with men as their risk for HIV infection.

10 patients had 12 baseline TDR mutations, including M46L (n=6), T215S (n=1), Y188L (n=1), L90M (n=1), M41L (n=1), M46I (n=1), I50I/L (n=1). Three mutations (M46L, I50I/L, M46L) in 3 patients (subject 22, 222, 25) were lost during the follow up, within 6 months after EDI. The total rate of replacement of baseline TDR was 25.0% (3 out of 12), the rate for M46L was 33.3% (2 out of 6), for I50L is 100.0% (1 out of 1) respectively, as shown in Table 1.

Quantify the variation of TDR mutations during the follow up in 5 acute/recent infected patients

HTS was used to quantify the variation of each TDR mutations during the follow up in 5 TDR positive patients (subjects 15, 22, 25, 42, 222).

Of the 3 patients (subject 22, 222, 25) whose mutations became undetectable by population sequencing, mutations (M46L) can be still be detected as minority variants in 2 of them (subject 222 and 25) at the end of follow-up (M24), but the amount has been decreased from 3%

to 2% and from 97% to 1% respectively. However, in subject 22, I50L mutation, which was 4% at baseline, could not be detected at the end of follow-up (M24) even by HTS.

Two additional low-frequencies HIV-1 drug resistance mutations disappeared at the end of follow-up by HTS, one is reverse transcriptase D67G mutants in subject 222, the other one is reverse transcriptase T215S mutants in subject 15, and the baseline percentage of both of the two mutations was 1%. All the other mutations seem to have a downward trend during the follow-up in percentage too. When we calculate absolute copies of each RAMs minority resistant viruses in patients, all mutations seemed also decline during the follow-up (Figure 1).

Minority mutations missed by Population-based sequencing and the discrepancies in viral susceptibility Prediction

The baseline genotype of 10 acute/early infected patients was included in assessing the discrepancies between population-based genotyping and HTS in TDR prevalence and viral susceptibility prediction. Among whom, five patients (subject 15, 22, 25, 42, 222) were TDR positive by population-based genotyping at the baseline, presenting aCRF01-AE subtype in 2 patients (40%) and a B subtype in 3 patients (60%), with a median of age at 27 (19-45), and presenting a median of 30 days (range, 17 to 50 days) after their EDI at diagnosis (baseline), with a median baseline CD4 count of 518 cells/mm³ (196-807) and 5.70 log₁₀ RNA copies/ml (3.51-7.78); The other 5 patients (subject 55, 64, 147, 199, 203) were TDR negative by population-based genotyping, presenting CRF01-AE subtype in 3 patients (60%) and B subtype in 2 patients (40%), with a median of age at 28 (25-33), and presented a median of 38 days (range, 14 to 87 days) after their EDI at diagnosis (baseline), with a median baseline CD4 count of 349 cells/mm³ (213-514) and 4.79 log₁₀ RNA copies/ml (4.37-5.68). The 10 patients were detected low-frequency HIV-1 drug resistance mutations by HTS.

Among the 5 positive samples (subject 15, 22, 25, 42, 222) population-based genotyping revealed 7 TDR mutations: M46L (n=3), T215S (n=1), Y188L (n=1), L90M (n=1), I50I/L (n=1). HTS confirmed all of the mutations detected by population sequencing, and detected an additional 8 minority RAMs in 3 of the 5 patients (60%): T215S (n=3), D67G (n=2), I50I/L (n=1), L90M (n=2), whose percentage in the viral quasi species ranged from 1% to 8%, modified 60.0% (3 out of the 5 patients) of phenotypic susceptibility predictions overall (Table 2 and Figure 2).

The additional T215S mutation discovered by HTS changed the predicted susceptibility of zidovudine and stavudine from sensitive to low-level resistance in subject 15 and subject 25. Furthermore, in subject 25 the detection of minority L90M mutants in protease by HTS in addition to the M46L detected by population sequencing, consistently decreased the virus susceptibility to nelfinavir, and changed the predicted susceptibility of indinavir/r, lopinavir/r, atazanavir/r, fosamprenavir/r, saquinavir/r from sensitive to low-level or intermediate resistance. In subject 222, the additional detection of the reverse transcriptase T215S and D67G mutants changed virus susceptibility to zidovudine, stavudine, abacavir, didanosine, tenofovir from sensitive to low-level or intermediate resistance. The detection of protease L90M and I50L mutants led to high-level resistance to PIs atazanavir/r, intermediate resistance to saquinavir/r, nelfinavir, low-level resistance to fosamprenavir/r, indinavir/r (Table 2 and Figure 2).

Subject	age	subtype	ETI	CD4 (cells/mm ³)	VL (log copies/mL)	NRTI	NNRTI	PI
ya92	23	CRF01-AE	D30	367	5.05	None	None	M46L
			M6	626	4.80	None	None	M46L
			M12	609	4.77	None	None	M46L
			M24	585	3.26	None	None	M46L
ya42	27	B	D17	330	7.78	T215S	Y188L	L90M
			M6	300	4.15	T215S	Y188L	L90M
			M12	340	4.14	T215S	Y188L	L90M
			M24	270	3.65	T215S	Y188L	L90M
			M51	106	4.20	T215S	Y188L	L90M
Ya15	30	B	D30	196	5.7	None	None	M46M/L
			M6	195	4.67	None	None	None
			M12	188	4.45	None	None	M46M/L
			M24	123	5.57	None	None	M46M/L
Ya34	46	CRF01-AE	D22	598	4.53	M41L	None	None
			M6	714	3.91	M41L	None	None
			M12	594	3.89	M41L	None	None
			M24	436	3.95	M41L	None	None
Ya231	24	CRF01-AE	D40	665	4.11	None	None	M46I
			M6	493		None	None	M46I
			M12	418	4.11	None	None	M46I
			M24	419		None	None	M46I
Ya18	43	CRF01-AE	D30	130	4.21	None	None	M46L
			M6	252	4.08	None	None	M46L
			M12	199	3.88	None	None	M46L
			M24	195	4.94	None	None	M46L
Ya95	24	CRF01-AE	D39	805	4.75	None	None	M46L
			M6	924	5.09	None	None	M46L
			M12	717	4.67	None	None	M46L
			M24	638		None	None	M46L
Ya22	25	B	D50	612	5.51	None	None	I50I /L
			M6	548	3.47	None	None	None
			M12	405	3.25	None	None	None
			M24	291	4.78	None	None	None
Ya25	45	CRF01-AE	D17	518	6.07	None	None	M46L
			M6			None	None	None
			M12			None	None	None
			M24	443	4.98	None	None	None
Ya222	19	CRF01-AE	D30	807	3.51	None	None	M46L
			M6	549		None	None	None
			M12	410	2.3	None	None	None
			M24	230		None	None	None

ETI, Estimated time of infection

Table 1: The TDR follow up of 10 acute/early patients (including 7 CRF01-AE subtype and 3 B subtype).

After 6 month-ART, all of the 5 patients got a virologic suppression. But subject 42, who had multiple resistance mutations (T215S, Y188L, L90M) did not show any increase of CD4 cell counts (250 cells/mm³ to 230 cells/mm³).

Of the 5 negative patients (subject 55, 64, 147, 199, 203), 13 minority RAMs in 4 of the 5 patients (80.0%) were found: T215S (n=3), K101E

(n=1), V82A (n=1), L90M (n=1), M184V (n=1), K219R (n=1), I47V (n=1), N83D (n=1), M41V (n=1), D67G (n=1), L210W (n=1), whose percentage in the viral quasi species ranged from 1% to 3%. Modified the phenotypic susceptibility predictions in 4 out of 5 patients (80.0%): in subject 55, HTS discovered reverse transcriptase M184V mutation and 2 thymidine analogues related mutations (TAMs) T215S and K219R, which predicted to be high level resistant to lamivudine and

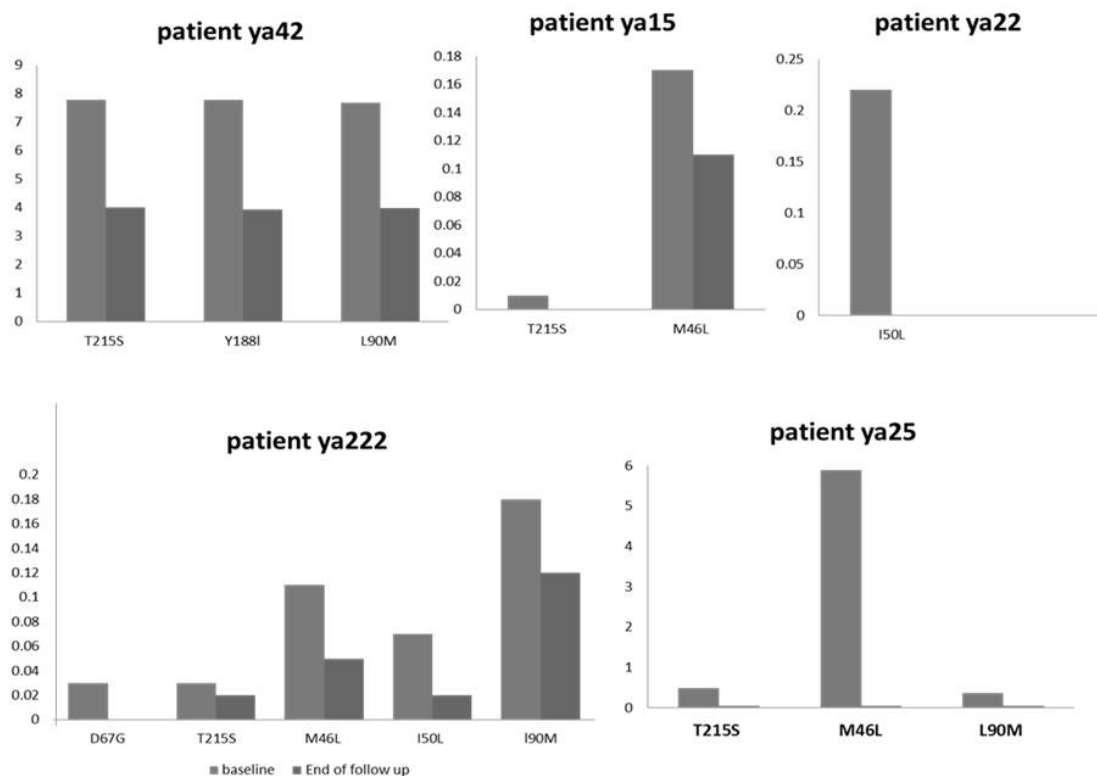


Figure 1: Decrease of TDR Mutations during the Follow Up in 5 acute/ recent infected patients. The y axis denotes the quantity of each mutation (log copies/mL), which was calculated by multiplying the percentage with viral load. The detection limit of population-based sequencing for drug-resistant HIV-1 is approximately 20%, which is based on general consensus and the cut-off value of the high-throughput sequencing (HTS) we used was 1%.

Patient	Timepoint	Genotype	EFV	NVP	RPV	ETR	AZT	D4T	FTC	3TC	ABC	DDI	TDF	ATV/r	DRV/r	FPV/r	IDV/r	LPV/r	NFV	SQV/r	TPV/r	ARV regimen	Virologic response after 6 months of treatment	
42	baseline	Population-based	Red	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	AZT+3TC+EFV	virologic suppression
15	baseline	Population-based	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	not done	
222	baseline	Population-based	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	TDF+3TC+EFV	virologic suppression
22	baseline	Population-based	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Red	Green	Green	Green	Green	Green	Green	Green	Green	AZT+3TC+EFV	virologic suppression
25	baseline	Population-based	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	not done	
55	baseline	Population-based	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	D4T+3TC+NVP	in complete virologic response
64	baseline	Population-based	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	D4T+3TC+NVP	virologic suppression
147	baseline	Population-based	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	AZT+3TC+EFV	virologic suppression
199	baseline	Population-based	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	TDF+3TC+EFV	virologic suppression
203	baseline	Population-based	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	AZT+3TC+EFV	in complete virologic response

Figure 2: Table showing level of resistance to the antiretroviral treatment. Red is "High-level resistance"; orange is "Intermediate resistance"; yellow is "Low-level resistance"; green is "Sensitive" or "Potential low-level resistance" virologic suppression. A confirmed HIV RNA level below the limit of assay detection; incomplete virologic response: Two consecutive plasma HIV RNA levels >200 copies/mL after 24 weeks on an ARV regimen.

Subject	Age	subtype	ETI	CD4 cells/mm ³	VLLog copies/mL	Mutation by PS	mutations by HTS	Change in predicted phenotype
Ya42	27	B	D17	330	7.78	NRTIs: T215S	NRTIs: T215S100%	-
						NNRTIs: Y188L	NNRTIs: Y188L100%	-
						PIs: L90M	PIs: L90M99%	-
Ya15	30	B	D30	196	5.7	NRTIs: none	NRTIs: T215S1%	AZT,D4T: iii
						NNRTIs: none	NNRTIs: none	-
						PIs: M46M/L	PIs: M46L3%	-
Ya222	19	CRF01-AE	D30	807	3.51	NRTIs: none	NRTIs:D67G1%, T215S1%	AZT,D4T: iv; ABC,DDI,TDF: iii
						NNRTIs: none	NNRTIs: none	-
						PIs: M46L	PIs: M46L2%, I50L3%, L90M5%	ATV/r: v; SQV/r, NFV: iv; FPV/r, IDV/r: iii
Ya22	25	B	D50	612	5.51	NRTIs: none	NRTIs: none	-
						NNRTIs: none	NNRTIs: none	-
						PIs:I50I /L	PIs:I50L4%	-
Ya25	45	CRF01-AE	D17	518	6.07	NRTIs: none	NRTIs: T215S8%	AZT,D4T: iii
						NNRTIs: none	NNRTIs: none	-
						PIs: M46L	PIs:M46L97%, L90M6%	NFV: v; SQV/r: iv; ATV/r,FPV/r,IDV/r: iii
Ya55	27	CRF01-AE	D87	349	4.79	NRTIs: none	NRTIs: M184V1%,T215S2%,K219R1%	3TC,FTC: v; ABC: iv; AZT,D4T,DDI: iii
						NNRTIs: none	NNRTIs: none	-
						PIs:none	PIs:none	-
Ya64	33	B	D60	481	4.37	NRTIs: none	NRTIs: none	-
						NNRTIs: none	NNRTIs: none	-
						PIs:none	PIs:none	-
Ya147	28	B	D38	514	4.73	NRTIs: none	NRTIs: none	-
						NNRTIs: none	NNRTIs: none	-
						PIs:none	PIs:I47V1%,N83D1%	TPV/r, FPV/r: iv; IDV/r,LPV/r,NFV: iii
Ya199	30	CRF01-AE	D14	213	5.39	NRTIs: none	NRTIs: T215S 2%	AZT,D4T: iii
						NNRTIs: none	NNRTIs:K101E 2%	NVP,RPV: iv; EFV: iii
						PIs:none	PIs:V82A1%,L90M 3%	NFV: v; ATV/r,FPV/r,IDV,LPV/ r,SQV/r: iv;
Ya203	25	CRF01-AE	D31	304	5.68	NRTIs: none	NRTIs:M41L1%,D67G1%,L210W1%, T215S 1%	AZT,D4T: v; ABC,DDI,TDF: iv;
						NNRTIs: none	NNRTIs: none	-
						PIs:none	PIs:none	-

Table 2: Minority mutations missed by Population-based sequencing and the discrepancies in viral susceptibility prediction.

emtricitabine, intermediate resistant to abacavir, low level resistant to zidovudine, stavudine and didanosine. In subject 203, additional discovered minority TAMs of M41L, D67G, L210W, T215S, predicted a significantly reduced sensitivity in all of the NRTIs drugs except for lamivudine and emtricitabine. In subject 199, T215S and K101E were detected, led to reduced sensitivity in zidovudine, stavudine and three important NNRTIs drugs: efavirenz, nevirapine, rilpivirine. However, the detection of minority protease V82A, L90M mutants caused a decrease in the virus susceptibility to nelfinavir, atazanavir/r, fosamprenavir/r, indinavir/r, lopinavir/r, saquinavir/r. In subject 147, only minority protease related mutants I47V, N83D were detected, leading to a decreased susceptibility to tipranavir/r, fosamprenavir/r, indinavir/r, lopinavir/r, nelfinavir (Tables 2 and 3 and Figure 1).

After 6 months of ART, subject 55 and subject 203 got an incomplete virologic response (the ARV regimen was D4T+3TC+NVP and AZT+3TC+EFV respectively), HIV RNA was 13386 copies/ml and 210 copies/ml, respectively. No CD4 cells increase was seen (290 cells/mm³ to 260 cells/mm³ and 300cells/mm³ to 280 cells/mm³) [20].

Discussion

Without the drug selection pressure, wild-type HIV will reappear rapidly in patients with secondary resistance, which has persisted in the cellular compartment [20]. But, such a rapid shift of TDR is unlikely after transmitted to a new host. Because of the “genetic bottleneck”, most HIV infections are initiated by a single variant [11]. That means, wild-type virus is rarely co-transmitted with drug-resistant variants,

Subject	Follow-up	baseline			End of follow up		
		Mutation by PS	mutations by HTS	Change in predicted phenotype	Mutation by PS	mutations by HTS	Change in predicted phenotype
Ya42	57M	NRTIs: T215S	NRTIs: T215S100%	-	NRTIs: T215S	NRTIs: T215S96%	-
		NNRTIs: Y188L	NNRTIs: Y188L100%	-	NNRTIs: Y188L	NNRTIs: Y188L94%	-
		PIs: L90M	PIs: L90M 99%	-	PIs: L90M	PIs: : L90M 95%	-
Ya15	24M	NRTIs: none	NRTIs: T215S 1%	AZT,D4T: iii;	NRTIs: none	NRTIs: none	-
		NNRTIs: none	NNRTIs: none	-	NNRTIs: none	NNRTIs: none	-
		PIs: M46M/L	PIs: M46L3%	-	PIs: M46M/L	PIs: M46L2%	-
Ya222	24M	NRTIs: none	NRTIs:D67G 1%, T215S 1%	ABC,DDI,TDF: iii; AZT,D4T: iv;	NRTIs: none	NRTIs: T215S 1%	AZT,D4T: iii
		NNRTIs: none	NNRTIs: none	-	NNRTIs: none	NNRTIs: none	-
		PIs: M46L	PIs: M46L3%, I50L 2%, L90M 5%	ATV/r: v; SQV/r, NFV: iv; FPV/r, IDV/r: iii;	PIs:none	PIs: M46L2%, I50L 1%, L90M 5%	ATV/r: v; SQV/r, NFV: iv; FPV/r, IDV/r: iii;
Ya22	24M	NRTIs: none	NRTIs: none	-	NRTIs: none	NRTIs: none	-
		NNRTIs: none	NNRTIs: none	-	NNRTIs: none	NNRTIs: none	-
		PIs:I50I /L	PIs:I50L4%	-	PIs:none	PIs:none	-
Ya25	24M	NRTIs: none	NRTIs: T215S 8%	AZT,D4T: iii;	NRTIs: none	NRTIs: T215S 1%	AZT,D4T: iii;
		NNRTIs: none	NNRTIs: none	-	NNRTIs: none	NNRTIs: none	-
		PIs: M46L	PIs: M46L 97%, L90M 6%	ATV/r,FPV/r,IDV/r: iii; SQV/r: iv; NFV: v;	PIs: none	PIs: M46L 1%, L90M 1%	ATV/r,FPV/r,IDV/r: iii; SQV/r: iv; NFV: v;

Table 3: Detection of minority mutations missed by direct sequencing and quantification of the variation of TDR mutations.

there is no 'memory' of the original wild-type in a new host. Given only drug-resistant variants are present in the new host, a novel starting point for viral evolution is created: nucleotide changes in the quasi species are modulated by chance and will be selected if they have a beneficial effect on viral fitness. Transmitted drug-resistant variants may persist or fade away from detection in the plasma depending on their relative fitness in the new environment.

Based on studies before, it was believed that three different evolutionary pathways exist. First, if the RAM has a profound effect on RC, it will revert to wild-type (including incomplete evolution) variants. A second pathway is evolution to atypical variants. Finally, persistence of drug-resistant variants may be observed due to several underlying mechanisms, such as a limited decrease in RC, compensatory fixation, and furthermore, selective pressure by the immune system may also influence the evolution or persistence of transmitted resistance profiles [21]. Marieke Pinggen et al. [21] reviewed 12 papers and summarized the evolutionary pathways of transmitted drug-resistant HIV-1 in 58 cases who were observed up to 58 months. In general, after the transmission, RT mutation M184V became undetectable by population sequencing and 215 mutations are replaced by other variants quickly. Mutations such as K103N and most other NNRTI resistance mutations often tend to persist. Within the extensive PR profiles, individual mutations occasionally reverted over time, but the majority of mutations persisted.

In our study, we longitudinally observed 10 acute/recent infected patients. The baseline mutations in these patients included RT T215S, Y188L, M41L and PT M46L/I, L90M, I50L. Over time, we observed a reversing rate of 25%. The reversion of resistance was observed in 3 cases, included 2 M46L mutations and 1 I50L mutation, all of the reversion happened within 6 months. One patient who was infected with viruses harboring T215S, Y188L, L90M mutations at the same time didn't show any change of the mutations pattern during 51 months' follow-up.

However, when we use HTS to quantify the variation of the

mutations, of the three mutations seem as reversion during the follow-up, only the RT M46L mutation in subject 25 decreased significantly (decreased from 97% percent at baseline to 1% percent at 24 months after ETI), the other two mutations, RT M46L mutation in subject 222 and PT I50L mutations in subject 22, which cannot be detected by population-based sequencing at 6 months after ETI, had a very low percentage (3% and 4%, respectively) at baseline. Given conventional population-based sequencing usually cannot detect the variants in the viral population present below 20%, the detection of these two mutations at baseline might be accidental. But anyway, all of the mutations showed a downward trend, not only in percentage but also in absolute copy numbers, during the follow-up measured. Even three low-frequent HIV-1 drug resistance mutations (RT D67G mutation 1% in subject 222, RT T215S mutant 1% in subject 15 and PT I50L mutation 4% in subject 22) disappeared at the end of follow-up.

Persistence of secondary drug-resistant virus (generated from inadequate treatment) has been well-documented not only in long-lived cellular reservoirs [22,23] but also in blood plasma as minority species several months up to years after treatment discontinuation, when conventional sequencing reports a complete reversion to wild-type virus [24,25]. Studies suggested that nevirapine resistance mutations identified in women and infants after single-dose nevirapine administration decline rapidly over time but can persist at low levels in the blood plasma up to 12-24 months after exposure before returning to basal background levels [26]. These reports raise a fundamental question of whether the TDR prevalence has been underestimated when measured by standard genotypic assays. As more sensitive sequencing techniques were introduced, the presence of minority variants in untreated patients was well documented. But where did these variants come from and their clinical implications are still controversial.

Theoretically, there are at least two sources of minority HIV-1 drug resistance mutations found in drug-naive patients: transmitted drug resistance or de novo generation as part of natural viral diversification. As mentioned above, compared to wild type HIV-1, viruses harboring

resistance mutations generally have lower fitness. After transmitted to the new host, without the drug-selective pressure, the frequency of such transmitted HIV-1 drug resistance mutations is likely to decay and at a certain time would no longer be detectable by current genotyping assays that rely on population sequencing [7,9]. In addition, HIV-1 minority variants can also arise due to the underlying diversity of the viral population. This remarkable diversity stems from a high replication rate and the error-prone reverse transcriptase enzyme. Because of this underlying diversity, it is estimated that, drug resistance mutations are likely to be present during chronic infection even in the absence of drug exposure, with the frequency of the mutations dependent on their fitness costs [27]. Gianella S et al. [28] performed Ultra Deep Sequencing (UDS) of partial HIV-1 gag, pol, and env genes from 32 recently infected individuals, differentiated biologically meaningful mutations from those caused by methodological errors, and examined the association between detected minority DRM and the virologic failure of first-line antiretroviral therapy (ART). They did not find any indication of increased diversity or selection at DRM sites compared to other sites no correlation between detected minority DRM and clinical failure of first-line ART. So they believed that the majority of low-frequency DRM detected using UDS are likely errors inherent to UDS methodology or a consequence of error-prone HIV-1 replication [28]. But the transmission of minority variants was supported by other reports. Metzner KJ et al. [29] quantified minority variants by allele-specific polymerase chain reaction in 204 acute or recent seroconverters and 382 ART-naive, chronically infected patients. They identified 12 potential transmitters from 16 acute or recent seroconverters harboring M184V minority variants. Second, prevalence between minority variants harboring the frequent mutation M184V and the particularly uncommon integrase mutation N155H differed highly significantly in acute or recent seroconverters. Third, the prevalence of less-fit M184V minority variants is significantly higher in acutely or recently than in chronically HIV-1-infected [29].

In our study we did see the downward trend of TDR in all acute/early infected patients we checked, and some of the mutations (such as RT M46L mutation in subject 25) turned from predominant strain to minority variants. Furthermore, we saw some related mutations in some of the patients. For example, in subject 55 and subject 203, multiple thymidine analogues related mutations were detected, which cannot be explained by virus replication errors. So we hypothesized, that TDR is at least one of the resources of minority variants.

Based on this hypothesis, it might be expected to have a higher prevalence of minority HIV-1 drug resistance variants in patients who have revertants and atypical variants. We found a report of a higher prevalence of minority variants in patients who were reported TDR by standard sequencing, where the investigators believe that the presence of TDR may be an indicator of a more extensive transmitted resistance profile [30]. But, we did not find a higher prevalence of minority variants in TDR positive patients than in TDR negative patients, the prevalence were both 80%. The reason might be multiple: there might be other sources (such as the consequence of error-prone HIV-1 replication) coexist, or the reversion of some TDR mutations happens really quickly after transmission, or they were transmitted as minority variants at the beginning.

Pooled analysis revealed that the pre-existing minority drug-resistant is associated with more than twice the risk of virologic failure [31]. It has been studied most rigorously for patients on an NNRTI-based first-line regimen in treatment-naive patients. For those with

NNRTI-resistant minority variants, it was found to have 1.6 times the risk of treatment failure compared to those without [31]. However, evidence has not yet emerged for a significant association between low-frequency PI resistance mutations and a significantly increased risk of treatment failure [32,33]. Although a single resistance mutation (I50L) confers high-level resistance to atazanavir, the significant reduction in viral fitness associated with this mutation may minimize the frequency and hence the impact of minority variants carrying this mutation [34].

In our study, the HTS revealed an additional 8 minority RAMs in 3 of the 5 patients (60%) who were reported TDR positive by population sequencing, modified 60.0% (3 out of the 5 patients) phenotypic susceptibility predictions of them. However, of the 5 patients who were reported TDR negative by population sequencing, HTS revealed an additional 13 minority RAMs in 4 of the 5 patients (80.0%), and modified the phenotypic susceptibility predictions in 4 out of 5 patients (80.0%). Of the two patients (subject 55 and subject 203) who did not get to virologic suppression after 6 months of ART, there was no TDR was found by population-based sequencing, but HTS found multiply thymidine analogues related mutations (in subject 55, T215S2%, K219R1% and M184V1%; in subject 203, M41L1%, D67G1%, L210W1%, T215S 1%). Although because of the small size of cases, we did not do any statistical analysis, it seems like the minority resistant variants can increase the risk of virological failure.

There are at least 2 ways by which pre-exist minority resistant variants contribute to virologic failure [33]. First, they can be selected directly with minimal evolution by the regimen, outgrowing wild-type variants to become the dominant mutant virus population during virologic failure. Palmer et al. [24] analyzed phylogenetic NNRTI resistant and wild-type sequences at entry and at the time of virologic failure and demonstrated tight clustering of NNRTI-resistant sequences in 2 of 11 patients. Second, although limited sequence sampling and recombination may explain the lack of clustering in some patients, it is also possible that minority drug-resistant variants contribute to virologic failure through providing a replicating virus population from which more-resistant viruses emerge.

Thus we conclude that the risk of virology failure depends on both the individual mutation and its frequency in the viral population. Other factors, such as treatment adherence, could also increase risks of treatment failure if mutations are present at very low levels.

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Author Contributions

LD - Conceived experimental design and laboratory and data analysis of the patients in the Youan-China Cohort and manuscript writing; SM - Data Analysis and manuscript writing; DS, BN - laboratory analysis; CBH - Patient data analysis SAS - Patient data analysis; NL - laboratory and data analysis of the patients in the Youan-China Cohort; HW - Study design and Youan Hospital Clinic in charge and Clinical evaluation of all patients in the Youan-China cohort.

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