Prevalence of WHO Transmitted Drug Resistance Mutations by Deep Sequencing in Antiretroviral-Naïve Subjects in Hunan Province, China

Zou Xiaobai1, Chen Xi1*, Hongping Tian2, Ann B. Williams3, Honghong Wang4, Jianmei He1, Jun Zhen1, Jennifer Chiarella5, Lisebeth A. Blake6, Gregory Turenchalk6, Michael J. Kozal5

1 Hunan Provincial Center for Disease Control and Prevention, Changsha, Hunan Province, China, 2 Yale-China Association, New Haven, Connecticut, United States of America, 3 UCLA School of Nursing, Los Angeles, California, United States of America, 4 Central South University, Hunan Province, China, 5 Yale School of Medicine, New Haven, Connecticut, United States of America, 6 454 Life Science, Branford, Connecticut, United States of America

Abstract

**Background:** There are few data on the prevalence of WHO transmitted drug resistance mutations (TDRs) that could affect treatment responses to first line antiretroviral therapy (ART) in Hunan Province, China.

**Objective:** Determine the prevalence of WHO NRTI/NNRTI/PI TDRs in ART-naïve subjects in Hunan Province by deep sequencing.

**Methods:** ART-naïve subjects diagnosed in Hunan between 2010–2011 were evaluated by deep sequencing for low-frequency HIV variants possessing WHO TDRs to 1% levels. Mutations were scored using the HIVdb.stanford.edu algorithm to infer drug susceptibility.

**Results:** Deep sequencing was performed on samples from 90 ART-naïve subjects; 83.3% were AE subtype. All subjects had advanced disease (average CD4 count 134 cells/mm3). Overall 25.6% (23/90) of subjects had HIV with major WHO NRTI/NNRTI TDRs by deep sequencing at a variant frequency level >1%; 16.7% (15/90) had NRTI TDR and 12.2% (11/90) had a major NNRTI TDR. The majority of NRTI/NNRTI mutations were identified at variant levels <5%. Mutations were analyzed by HIVdb.stanford.edu and 7.8% of subjects had variants with high-level nevirapine resistance; 4.4% had high-level NRTI resistance. Deep sequencing identified 24(27.6%) subjects with variants possessing either a PI TDR or hivdb.stanford.edu PI mutation (algorithm value ≥15). 17(19.5%) had PI TDRs at levels >1%.

**Conclusions:** ART-naïve subjects from Hunan Province China infected predominantly with subtype AE frequently possessed HIV variants with WHO NRTI/NNRTI TDRs by deep sequencing that would affect the first line ART used in the region. Specific mutations conferring nevirapine high-level resistance were identified in 7.8% of subjects. The majority of TDRs detected were at variant levels <5% likely due to subjects having advanced chronic disease at the time of testing. PI TDRs were identified frequently, but were found in isolation and at low variant frequency. As PI/r use is infrequent in Hunan, the existence of PI mutations likely represent AE subtype natural polymorphism at low variant level frequency.

Citation: Xiaobai Z, Xi C, Tian H, Williams AB, Wang H, et al. (2014) Prevalence of WHO Transmitted Drug Resistance Mutations by Deep Sequencing in Antiretroviral-Naïve Subjects in Hunan Province, China. PLoS ONE 9(6): e98740. doi:10.1371/journal.pone.0098740

Editor: Luis Menéndez-Arias, Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Spain

Received September 19, 2013; Accepted May 7, 2014; Published June 4, 2014

This is an open-access article, free of all copyright, and may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose. The work is made available under the Creative Commons CCO public domain dedication.

Funding: This work was supported by a grant from Abbvie (Kozal, PI) and by U.S. NIH R34 MH 083564 (Williams, PI). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. This does not alter the authors’ adherence to all the PLOS ONE policies on sharing data and materials.

Competing Interests: Yale University receives grant support from Merck, Pfizer, ViIV, Vertex, Gilead, Abbott, Glaxo Smith Kline, Hologic Inc., and Bristol-Myers Squibb for studies that Dr. Kozal serves as the principal investigator. Dr. Kozal is a federal employee and does not receive grant support directly – the grant is held by Yale University. Dr. Kozal receives royalties from a patent owned by Stanford University for some HIV genotyping tests. The COI reported by Dr Kozal does not alter the authors’ adherence to all the PLOS ONE policies on sharing data and materials. Lisabeth Blake and Gregory Turenchalk are employees of Roche-454 Life Sciences and their participation does not alter the authors’ adherence to all the PLOS ONE policies on sharing data and materials.

* E-mail: Chenxi161@sohu.com

Introduction

Hunan is the 11th largest province of China, situated in the southeast region of the country. Commercial sex work and injection drug use are highly prevalent; and the HIV/AIDS epidemic has expanded rapidly. Through the end of 2012 more than 16,000 HIV-infected cases have been reported in Hunan Province [1–4]; Hunan HIV epidemiology and treatment data-base system. More than 6,600 HIV-infected patients are in care and have received free antiretroviral therapy (ART) which is supported by the government. Currently, 5,133 patients remain on ART (Hunan HIV epidemiology and treatment database system). Prior to 2012 in Hunan Province, the first line ART consisted of triple therapy with 2 nucleos(t)ide reverse transcriptase inhibitors (NRTIs) with a non-nucleoside reverse transcriptase inhibitor (NNRTI) - available first line agents were: stavudine (d4T),
considered unlikely.

system, misrepresentation of treatment history by subjects was ART. As ART is provided exclusively through the China CARES

PLOS ONE | www.plosone.org 2 June 2014 | Volume 9 | Issue 6 | e98740

Setting and Subjects

Materials and Methods

Ethics Statement

The study was conducted at two clinical sites, in Hengyang City and Changsha City, Hunan Province. At each site, clients receive comprehensive evaluation and ART when indicated. The study was approved by the Human Investigative committees/IRBs at Yale University, New Haven, CT, USA and Central South University, Hunan Province, China and all subjects in the study gave written informed consent.

Setting and Subjects

Throughout China and in Hunan, the national China CARES program provides clinical evaluation and free medication to HIV-infected individuals. Patients attending the China CARES clinics are poor and primarily rural residents. All individuals presenting for initial anti-retroviral therapy at one of the two largest China CARES sites (Changsha and Hengyang) in Hunan between July 5, 2010 and August 8, 2011 were invited to participate in this descriptive cross sectional study of HIV-1 RNA quantity and genotype. The two study sites provide care to 35.9% (1065/2970) of provincial China CARES patients.

All subjects were 18 years of age or older, understood and spoke Mandarin, and were mentally competent to answer questions in the judgment of the clinic physician. All invited individuals had CD4 counts below 350 cells/mm³ and had not previously received ART. As ART is provided exclusively through the China CARES system, misrepresentation of treatment history by subjects was considered unlikely.

Two hundred thirty-seven individuals were invited to participate; 20 declined. Plasma samples were obtained from the 217 consenting individuals before beginning ARV and stored at the

WHO HIV TDRs by Deep Sequencing in Hunan Province China

lamivudine (3TC), zidovudine (AZT), tenofovir (TDF) and nevirapine (NVP). Boosted protease inhibitors (PI/r) have been used infrequently in Hunan. Lopinavir/ritonavir (LPV/r) could be used in second line regimens; however, there has not been wide scale use of this agent in Hunan Province as of yet. Routine HIV viral load (VL) testing is generally performed once a year in patients on ART.

Although ART is very successful in treatment HIV/AIDS, the efficacy of first line regimens can be reduced by the presence of transmitted drug resistance mutations (TDRs). There are few data on the prevalence of World Health Organization (WHO) TDRs that could affect treatment responses to the first line regimen for ART-naive HIV-infected persons in Hunan. HIV subtype AE predominates in Hunan, but data on the prevalence of low frequency HIV variants with NRTI, NNRTI and PI resistance mutations among this subtype are limited. Currently available conventional genotyping sequencing does not detect low-level resistant variants at levels less than 20% of the circulating viral quasispecies [5]. There are reports that low abundance drug resistant HIV variants at levels as low as 1% of the circulating viral quasispecies can be detected in ART-naive individuals by more sensitive and quantitative genotyping technologies [5–20]. Low-frequency NNRTI drug-resistant variants below the 20% level have been strongly associated with virologic failure for subjects initiating NNRTI-based therapy [16–20].

In this study, we investigated the prevalence of WHO NRTI and NNRTI TDRs that can affect the treatment responses to first line ART among ART-naive HIV infected subjects entering care in Hunan Province, China. In addition, we determined the prevalence of WHO TDR and Stanford.HIVdb.edu PI resistance mutations in ART-naive subjects.

Materials and Methods

CD4+ T cells absolute count and Viral load testing

CD4+ T cell count was measured by flow cytometry (FACS Calibur, BD Bioscience, USA). HIV-1 RNA viral load was quantified with the COBAS AmpliPrep COBAS TaqMan HIV-1 test (Roche Diagnostics Systems), version 2.0 (CAP/CTM v2.0) with a lower limit of detection of 40 RNA c/ml.

Deep Sequencing

Samples from ART-naive subjects were evaluated by deep sequencing [16,21–25] for low-frequency HIV variants possessing WHO TDRs to 1% variant levels. Plasma samples were collected before starting therapy and stored in aliquots at −80°C until processed for deep sequencing. HIV-1 viral RNA was extracted from 140 ul of plasma samples using QIAamp RNA Mini Kit (Qiagen, Germany). The average plasma HIV viral load was 17,137 c/ml (median 73,600 c/ml; range 3,980 to 1,560,000 c/ml). Deep sequencing was performed at Roche Application Support Center Laboratory in Shanghai, China. The extracted viral RNA was sent to the application support center (Roche Applied Science, Asia Pacific) for deep sequencing with 2 ug of the included polyA-RNA carrier.

Dried down proprietary sets of specific primers targeted to HIV pol were used to generate 4 overlapping amplicons at ~400 bp (see Table S1 for details). cDNA synthesis and the amplicon generation was performed on 10 samples in a microtiter plate with specific primers targeted to four overlapping gene regions, encompassing about 1.3 kb sequence of the HIV pol region, include protease (PR) and reverse transcriptase (RT) genes. Four amplicons (~400 bp in length) were amplified for each sample, and the fusion primers contained the Roche 454 amplicon adaptor sequences and multiplex identifier (MID) tags on both forward and reverse primers (see Table S1a for plate layout and Table S1b and Table S1c for primer sequences). Each amplicon was purified with Agencourt AMPure XP magnetic beads (Beckman Coulter, Beverly, MA, US) at a bead:DNA volume ratio of 0.81; and quantified by PicoGreen Fluorescence using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Life Technologies, US). Agilent 2100bioanalyzer (Agilent Life Science, Santa Clara, California, US) was used to verify the quality and length of the amplicons. After quality controls, each set of 40 amplicons from a group of 10 samples were pooled, amplified by emPCR and sequenced in one GS Junior (454 Life Sciences, Roche, Branford, CT, US) run according to the manufacturer’s sequencing protocol. Amplicons were pyrosequenced in both forward and reverse directions, with read depths >1000X (RT IQR 3,313–9,180 and PI IQR 3,356–9,152).

The sequence reads were analyzed with the GS Amplicon Variant Analyzer (AVA) software (Roche) which assigns each read to the proper amplicon and patient sample, using the multiplex identifier (MID) tags. AVA software was used for mapping and calculating variant frequencies at each nucleotide position relative to the sequence of HIV-1 reference strain HXB2 sequence and Drug Resistant Mutations (DRMs) were counted according to a predefined DRMs list [26]. Deep sequencing reproducibility and level of variant detection have been previously reported...
Resistance Mutation Detection and Analysis

The full deep sequencing results are available through http://www.ncbi.nlm.nih.gov/sra; submission ID: Hunan CDC: 454 HIV Hunan China PRJNA248226; sample numbers: SRS579392; SRS579686; SRS579687–SRS579774 [link to metadata: ftp://ftp-trace.ncbi.nlm.nih.gov/sra/review/SRP040396_20140325_173500_bca7ba75caac67b75fa8c9a101f25bf1].

All amino acid positions associated with antiretroviral resistance according to the 2009 edition of the WHO Transmitted Drug Resistance Mutations (TDRs) list [26] and Stanford HIV drug resistance database (hivdb.stanford.edu) were evaluated [27]. WHO list emphasizes mutations that are thought not to be polymorphic and therefore more likely to be TDRs. The Stanford resistance database includes all polymorphic mutations that may contribute to low levels of drug resistance. WHO TDRs for RT: L210W, T215Y/F/I/S/C/D/V/E, K219Q/E/N/R [26].

Identified mutations were also interpreted using the HIVdb-stanford.edu algorithm to infer drug susceptibility and 18.9% of subjects (17/90) had viral variants at levels >1%. Of those with and without a WHO TDR is presented in Table 4. In addition, Sanger sequencing of RT was successful in 89/90 subjects. Three subjects had HIV with WHO TDR by Sanger sequencing (all with RT T69N) that were missed by deep sequencing.

To qualify for ART in 2010–2011 in this region a subject had to have a CD4 count <350 cells/mm³. Thus all patients deep sequenced had advanced disease and were likely chronically infected. Demographic, disease stage and virologic information for those with and without a WHO TDR is presented in Table 4. Given that this was a pilot study the data is presented descriptively as it was designed to determine prevalence of WHO TDRs and not to examine associations between groups.

Deep Sequencing for HIV PI drug resistance

Deep sequencing for PI mutations was successful with 87 of 90 (96.7%) unique ART-naive patient samples ~83% were subtype AE. Among 87 ART-naive subjects, 24 (27.6%) had a viral variant with either a WHO TDR or Stanford.hivdb.edu PI mutation (algorithm value ≥ 15), Table 5. 17(19.5%) had WHO PI TDRs at levels >1%; V32I, M46L, I47V, G48V, I50V, F53L, I54T, Q58E, and V82T/A (variant levels range 1.04 to 4.6%). Stanford.hivdb.edu PI mutations were identified in samples from 21 subjects

| HIV Type | Number of Subjects with a WHO TDR by Deep Sequencing*. |
|----------|---------------------------------------------------------|
| NNRTI    | 11 (12.2%)                                             |
| NRTI     | 15 (16.7%)                                             |
| NNRTI or NRTI | 23 (25.6%)                                      |

*Number of subjects with a major WHO NNRTI or NRTI TDRs by deep sequencing at a HIV variant frequency level >1%. Percent (%) based on a total of 90 subjects with a deep sequencing result. WHO TDRs as listed by Bennett and colleagues (26).

doi:10.1371/journal.pone.0098740.t002
The most frequent and highest HIV variant level identified was T74S in 10 subjects (11.5%); T74S has a value of 15 only for nelfinavir. All low-level variants were interpreted by hivdb.stanford.edu, 8(9.2%) subjects had HIV variants with intermediate or high-level PI resistance (driven by V32I, I47V, G48V, I50V & V82A/T); 2 (2.3%) subjects had HIV variants with multiple PI mutations. Sanger sequencing identified 3 additional subjects had HIV with WHO PI TDRs (I50I/V, I54I/T, N88D/N) that were missed by deep sequencing. Overall 3 subjects had virus with dual class TDR at a HIV variant frequency level $<1%. No subject had triple class resistance detected.

### Discussion

This study aimed to elucidate the prevalence of HIV with WHO TDRs by deep sequencing in ART-naïve subjects, since TDRs may affect the efficacy of the first line ART in Hunan Province, China. We identified that the predominant HIV subtype was CRF01_AE (83.3%, 75/90). This result is similar to that previously reported from Hunan Province molecular epidemiology HIV survey (2009–2012) which reported that 4 HIV-1 subtypes are circulating in Hunan (subtypes AE, BC, B and C) and that CRF_01AE was the dominant subtype representing more than 70% of the infections [1–4]; Hunan HIV epidemiology and treatment database system).

Among the 90 ART-naïve subjects evaluated in this study, 25.6% of subjects harbored HIV variants possessing WHO NRTI/NNRTI TDRs by deep sequencing that would affect the efficacy of the first line ART used in the region. The most frequently identified TDRs present at $<1% levels were D67G/N, M184I, K65N/R, T215I/S, P225H, Y188C/H, K101E, V106A, and G190 A/E. All of the RT TDRs identified by deep sequencing were also found in another HIV drug resistance mutation study that used standard genotyping in Hunan province in 2009 [4]. Both these studies suggest that WHO TDR affecting first line therapy are circulating in Hunan Province.

The mutations identified in our study are ones that can be selected for by the ARTs in wide use in Hunan (d4T, ZDV, TDF, 3TC, and NVP). Of note is that the most common NNRTI mutations identified in this study are frequently selected for by NVP. The most common TDR mutation in Western countries (NNRTI – K103N mutation) where subtype B predominates was noticeably absent in our survey. The most common NNRTI TDR

| No. | WHO RT TDR >1% | WHO TDR Class >1% Variant level | STANFORD RT TDR>1% | STANFORD HIVdb* |
|-----|----------------|---------------------------------|-------------------|-----------------|
| 1001A | M184I(1.36%) | NRTI | M184I(1.36%), V179D(1.22%) | H-NRTI |
| 1014A | V106A(4.94%) | NRTI | V106A(4.94%) | H-NMRTI (NVP), I |
| 1022A | M41L(2.41%) | NRTI | M41L(2.41%), V179D(85.25%) | S, PL |
| 1051A | P255H(1.1%) | NRTI | P255H(1.1%) | I, LL |
| 3006A | K65R(1.5%), T215S(2.04%) | NRTI | K65R(1.5%), T215S(2.04%), T69N(1.18%), V179D(2.71%), V179D(1.02%), T215S(2.04%) | H-NRTI, I |
| 3013A | V188C(1.02%) | NNRTI | V188C(1.02%) | H-NMRTI (NVP) |
| 3019A | P225H(1.32%) | NNRTI | P225H(1.32%) | I, LL |
| 3021A | M184I(1.17%) | NNRTI | M184I(1.17%) | I, LL |
| 3024A | D67G(1.79%), G190E(3.36%) | NNRTI, NRTI | D67G(1.79%), V75I(1.82%), V179D(2.41%), V179D(5.28%), V179D(3.72%), G190E(3.36%), H221Y(1.59%) | H-NMRTI (NVP) |
| 3029A | D67N(1.15%) | NRTI | D67N(1.15%) | LL |
| 3058A | T215S(1.99%), K65N(8.13%) | NRTI | K65N(8.13%), V179D(25.77%), T215S(1.99%) | PL, LL |
| 3061A | Y188C(1.27%) | NNRTI | T69N(1.67%), Y188C(1.27%) | H-NMRTI (NVP) |
| 3079A | D67G(3.74%) | NRTI | D67G(3.74%) | LL |
| 3088A | K101E(1.42%) | NNRTI | K101E(1.42%), V179D(10.56%), F227L(1.35%) | I |
| 3099A | K219Q(3.24%) | NNRTI | K219Q(3.24%) | PL |
| 3096A | D67G(1.03%) | NRTI | D67G(1.03%) | LL |
| 3098A | P225H(1.69%) | NNRTI | T69N(66.55%), P225H(1.69%) | I, LL |
| 3106A | L74V(7.63%) | NRTI | L74V(7.63%) | H-NRTI, I |
| 3132A | Y188H(1.63%) | NNRTI | V179D(3.95%), Y188H(1.63%) | H-NMRTI (NVP) |
| 3134A | D67G(3.04%), Y188H(16.67%) | NNRTI, NRTI | D67G(3.04%), V179D(7.75%), Y188H(16.67%) | H-NMRTI (NVP) |
| 3138A | F77L(2.4%), G190A(1.41%) | NNRTI, NRTI | K70T(1.18%), F77L(2.4%), E138K(1.73%), V179T(6.1%), V179D(1.56%), G190A(1.41%) | H-NMRTI (NVP) |
| 3146A | D67G(4.45%) | NRTI | D67G(4.45%) | LL |
| 3157A | D67G(10.83%) | NRTI | D67G(10.83%), T69N(1.05%) | LL |

*Stanford HIVdb algorithm interpretation abbreviations: H – High level resistance; I – Intermediate level resistance; LL – Low level resistance; PL – potential low level resistance. NVP - Nevirapine. High level resistance conferring mutations are in Bold. World Health Organization (WHO) Transmitted drug resistance mutation (TDR) [26]. TDR reported if the viral variant level $>1% by deep sequencing.

doi:10.1371/journal.pone.0098740.t003
80% of the subjects are infected with subtype AE were: K101E, V106A, Y188C/H, and G190E/A. K101E and Y188C/H cause intermediate or high-level resistance to nevirapine (NVP) and low level resistance to efavirenz (EFV); V106A and G190A/E cause high level resistance to NVP and EFV [27]. We identified one subject with a L74V at a HIV variant level of 7.6%. The L74V RT mutation reduces susceptibility to ddI and ABC. ddI and ABC were not used in Hunan province, and the presence of this variant may represent a TDR acquired in another region or country. Sanger sequencing identified additional TDRs in a few subjects, however, different PCR primer sets were used for Sanger sequencing and the detection of additional mutations was likely due to the preferential amplification of different variants by the different primer sets.

Many of the mutations identified were at low levels, 5%. It is likely that the majority of the subjects in this study had been infected for years as subjects had to have a CD4 counts below 350 cells/mm³ to receive ART through the national China CARES program during this time period. Given that these subjects were likely chronically infected (the majority had CD4 counts $\geq 200$ cells/mm³) prior to presenting for ART initiation it may be that the mutant variants declined in frequency levels in relation to more fit wild type viruses over time. This has been noted in other studies [5,17–19]. There were some subjects that had mutations at higher levels e.g. K65N, V179D/E, V106I, and Y188H.

Agents currently available for triple therapy in Hunan Province China are d4T, ZDV, TDF, 3TC, NVP, efavirenz (EFV), and lopinavir/ritonavir (LPV/r).

PI TDR mutations were identified frequently by deep sequencing (24% of subjects), but were found in isolation and at low variant frequency. The most frequent and highest HIV variant level identified was T74S in 10 subjects (11.5%); T74S has a value of 15 only for nelfinavir. All low-level variants were interpreted by Stanford.hivdb.edu, 9.2% of subjects had intermediate or high-level

| Characteristic | With WHO RT TDR $n=23$ (%) | Without WHO RT TDR $n=67$ (%) | Overall N = 90 |
|----------------|-----------------------------|-----------------------------|----------------|
| Gender | | | |
| Male | 17(73.9%) | 48(71.6%) | 65 |
| Female | 6(26.1%) | 19(28.4%) | 25 |
| Marriage | | | |
| Married | 12(52.2%) | 32(47.8%) | 44 |
| Single | 11(47.8%) | 35(52.2%) | 46 |
| Age | | | |
| 20–29 | 4(17.4%) | 18(26.9%) | 22 |
| 30–39 | 10(43.5%) | 19(28.4%) | 29 |
| 40–49 | 5(21.7%) | 22(32.8%) | 27 |
| 50–59 | 1(4.4%) | 7(10.4%) | 8 |
| 60– | 3(13.0%) | 1(1.5%) | 4 |
| CD4 cell abs count(cells/mm³) | | | |
| 0–199 | 15(65.2%) | 47(70.1%) | 62 |
| 200–349 | 8(34.8%) | 20(29.9%) | 28 |
| ≥350 | 0(0.0%) | 0(0.0%) | 0 |
| HIV RNA median (copies/mL) | 77100 | 73300 | 73600 |
| Infection of the patients’ spouse | | | |
| Negative | 5(21.7%) | 12(17.9%) | 17 |
| Positive | 6(26.1%) | 14(20.9%) | 20 |
| No spouse | 8(34.8%) | 25(37.3%) | 33 |
| Unrevealed | 4(17.4%) | 16(23.9%) | 20 |
| Route of transmission | | | |
| MSM | 2(8.7%) | 8(11.9%) | 10 |
| IDU | 8(34.8%) | 15(22.4%) | 23 |
| Heterosexual | 13(56.5%) | 42(62.2%) | 55 |
| IDU and heterosexual | 0(0.0%) | 1(1.5%) | 1 |
| Unrevealed | 0(0.0%) | 1(1.5%) | 1 |
| WHO staging | | | |
| I | 12(52.2%) | 48(71.6%) | 60 |
| II | 1(4.3%) | 3(4.5%) | 4 |
| III | 10(43.5%) | 15(22.4%) | 25 |
| IV | 0(0.0%) | 1(1.5%) | 1 |

Table 4. Baseline Characteristics of HIV-1 infected patients stratified by WHO RT TDR.

doi:10.1371/journal.pone.0098740.t004
PI resistance (driven by V32I, I47V, I50V & V82A/T); only 2% of subjects had HIV variants with multiple PI mutations. As PI/r use is infrequent in Hunan, the existence of these PI mutations likely represent AE subtype natural polymorphism at low variant level frequency. Further deep sequencing studies are needed to better understand the prevalence of low-frequency variants possessing resistance mutations in AE subtype to better inform resistance algorithms and epidemiologic studies.

Our study has several limitations. First, in this pilot study we only evaluated 90 subjects by deep sequencing. This is a large number compared to other reports using deep sequencing, but the prevalence estimates would be better refined with a greater number of samples across multiple years. Second, we only reported low level variants to 1% levels. The 1% cut off was chosen because it is well established that low level variants for NNRTI TDRs have clinical significance if a patient initiates a NNRTI based regimen [16–20]. Clonal mixing experiments were not performed as part of this study but there are reports that 454 deep sequencing can detect low level variants to 1% levels with good accuracy [11,16,18,21–25,28–35]. This level is above the known error rates for PCR and pyrosequencing, although errors occurring in early rounds of amplification can still affect the results; and the 1% level has been reported in prior TDR prevalence studies using deep sequencing [11,16,18,21–25,28]. Lastly, we cannot correlate the low level variant detection with clinical outcome as that was not the aim of this pilot study. In the future, we plan to investigate the effect of low level variants possessing TDR specifically in subtype AE on virologic response rates with the first line ART used in China.

**Conclusions**

Using deep sequencing, we found that ART-naïve subjects from Hunan Province, China, who were infected predominantly with subtype AE, frequently possessed HIV variants with WHO NRTI/NNRTI/PI TDRs that would affect the first line ART used in the region. Specific mutations conferring high-level resistance to nevirapine were identified in 7.8% of the subjects. Many of the TDRs identified were at low variant levels (<5%), and may have been due to subjects having advanced chronic disease when tested (the majority had CD4 counts <200 cells/mm³ when presenting to initiate ART). This delay may have allowed time for mutant variants with TDRs to decline in frequency levels in relation to more fit wild type viruses. Ongoing surveillance is needed to monitor for TDRs in newly and chronically infected subjects in Hunan Province, China.

**Supporting Information**

Table S1 Table S1a. Plate Layout for HIV deep sequencing. Table S1b. Forward primer sequences. Table S1c. Reverse primer sequences.

---

**Table 5. HIV drug resistance PI mutation detection by deep sequencing.**

| No. | WHO PI TDR>1% | STANFORD PI TDR>1% | Stanford Mutation* Level |
|-----|---------------|---------------------|--------------------------|
| 1001A | none | T74S(2.23%) | L |
| 1007A | none | T74S(11.66%) | L |
| 1014A | none | T74S(1.66%) | L |
| 1017A | none | T74S(2.41%) | L |
| 1025A | V82T(1.64%) | V82T(1.64%),(V82I(1)(99.96%),(V82I(3)(2.08%) | I |
| 1032A | V32I(4.6%) | V32I(4.6%),T74S(4.73%) | I |
| 1038A | I50V(1.87%) | I50V(1.87%) | H |
| 1047A | Q58E(1)(2.64%) | Q58E(2.64%) | L |
| 1048A | F53L(1.04%) | F53L(1.04%) | L |
| 1055A | G48V(1.41%) | G48V(1.41%) | H |
| 1064A | G48V(1.27%) | G48V(1.27%) | H |
| 3009A | I47V(1.09%) | I47V(1.09%) | I |
| 3012A | F53L(1.04%) | F53L(1.04%), T74S(40.56%) | L |
| 3019A | I50V(1.41%) | I50V(1.41%), T74S(5%) | H |
| 3025A | I54T(1.79%) | I54T(1.79%) | L |
| 3039A | none | T74S(99.81%) | L |
| 3042A | none | T74S(99.37%) | L |
| 3058A | none | T74S(9.7%) | L |
| 3074A | V82A(1.56%) | V82A(1.56%) | L |
| 3078A | F53L(2.61%) | F53L(2.61%) | L |
| 3088A | M46I(2.8%) | M46I(2.8%) | L |
| 3128A | M46I(1.5%) | M46I(1.5%) | L |
| 3132A | M46I(1.38%) | M46I(1.38%) | L |
| 3134A | M46I(3.58%) | M46I(3.58%) | L |

*Stanford HIVdb algorithm interpretation abbreviations: H – High level resistance; I – Intermediate level resistance; L – Low level resistance; PL – potential low level resistance. HIVdb.stanford.edu [27] PI scores accessed March 21, 2014. World Health Organization (WHO).
doi:10.1371/journal.pone.0098740.t005
References

1. Chen X, Xing H, He JM, Zheng J, Zou XB, et al. (2008) Study on the threshold of HIV-1 drug resistance in Hunan province. Chin J Epidemiol. 29(6):787–789.

2. Chen X, Xing H, He JM, Zheng J, Zou XB, et al. (2005) A molecular epidemiological study on HIV-1 infection in Hunan province. Practical preventive medicine. 12(3): 483–485.

3. He JM, Chen X, Zheng XH, Zhong P, Zou XR, et al. (2007) Longitudinal survey of antiretroviral drug resistance among untreated HIV-1 infection individuals in Hunan province. Practical preventive medicine. 14(4): 1260–1262.

4. Zou XR, He JM, Zhang GQ, Li NZ-Chen X, et al. (2010) Drug resistance analysis on AIDS patients after highly active antiretroviral therapy in Hunan province. Clin J Infect control. 9(5): 305–309.

5. Panel on antiretroviral guidelines for adults and adolescents (2013) Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Department of Health and Human Services. Available: http://aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf. Accessed September 4, 2013.

6. Kapoor A, Jones M, Shafer RW, Rhee SY, Kazanjian P, et al. (2004) Detection of minority populations of HIV-1 expressing the K103N resistance mutation in patients failing nevirapine. J Acquir Immune Defic Syndr 38(1):37–42.

7. Metzner KJ, Rauch P, Walter H, Boesecke C, Zollner B, et al. (2005) Detection of minor drug resistant patients of HIV-1 in acute seroconverters. AIDS 19(16): 1819–1825.

8. Palmer S, Bolzt V, Maldarelli F, Kearney M, Halvas EK, et al. (2006) Selection and persistence of non-nucleoside reverse transcriptase inhibitor-resistant HIV-1 in patients starting and stopping non-nucleoside therapy. AIDS 20(3): 701–710.

9. Roquebert B, Malet I, Wirden M, Tubiana R, Valantin MA, et al. (2006) Role of antiretroviral-Naı¨ve Patients Significantly Impact Treatment. J Infect Dis 199(5): 693–701.

10. Metzner KJ, Rauch P, Walter H, Boesecke C, Zollner B, et al. (2005) Detection of minority populations of HIV-1 expressing the K103N resistance mutation in patients failing nevirapine. J Acquir Immune Defic Syndr 38(1):37–42.

11. Lecossier D, Shulman NS, Morand-Joubert L, Shafer RW, Joly V, et al. (2005) Detection of minority populations of HIV-1 expressing the K103N resistance mutation in patients failing nevirapine. J Acquir Immune Defic Syndr 38(1):37–42.

12. Korn K, Reil H, Walter H, Schmidt B (2003) Quality Control Trial for Human

13. Disposition

14. Acknowledgments

15. Author Contributions

16. Conceived and designed the experiments: ZX CX HT AW HW JC MK. Performed the experiments: ZX CX. Analyzed the data: ZX CX HT AW HW JC LAB GT MK. Contributed reagents/materials/analysis tools: ZX CX LAB GT JC MK. Wrote the paper: ZX CX HT AW HW JC MK.