ORIGINAL ARTICLE

In-house human immunodeficiency virus–1 genotype resistance testing to determine highly active antiretroviral therapy resistance mutations in Hong Kong

Jonathan HK Chen 陳漢坤
KH Wong 黃加慶
Patrick CK Li 李頌基
Kenny KC Chan 陳志偉
MP Lee 李文寶
Sabrina WC To 杜維之
WC Yam 任永昌

Objective To determine the frequency of highly active antiretroviral therapy resistance mutations in the viral *pol* gene of human immunodeficiency virus–1 (HIV-1) genotypes that circulate in Hong Kong, by means of an in-house HIV-1 genotyping system.

Design Retrospective study.

Setting Two HIV clinics in Hong Kong.

Patients A modified in-house genotyping resistance test was used to sequence the partial *pol* gene in 1165 plasma samples from 965 patients. The performance of our test was cross-compared with the US Food and Drug Administration–approved ViroSeq HIV-1 genotyping system. The results of genotyping were submitted to the Stanford HIV-1 drug resistance database for analysis.

Results The cost-effective in-house genotypic resistance test (US$40) demonstrated comparable performance to the US Food and Drug Administration–approved ViroSeq system. The detection limit of this in-house genotypic resistance test could reach 400 copies/mL for both HIV-1 subtype B and CRF01_AE, which were the predominant genotypes in Hong Kong. Drug resistance mutations were detected only in post-treatment samples from treatment-failure patients. However, there was no significant difference in the frequency of drug resistance mutations between subtype B and CRF01_AE.

Conclusion Our cost-effective in-house genotypic resistance test detected no significant difference in drug resistance–related mutations frequencies between HIV-1 subtype B and CRF01_AE in Hong Kong. A drug resistance–related mutations database for different HIV-1 genotypes should be established in Hong Kong to augment guidance for HIV treatment.

Key words
Antiretroviral therapy, highly active; HIV-1; Drug resistance, viral; Genotype; Mutation, missense

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New knowledge added by this study

- The newly developed test was applicable to various human immunodeficiency virus–1 (HIV-1) genotypes prevailing in Hong Kong.

Implications for clinical practice or policy

- Use of the in-house HIV-1 genotyping resistance test is recommended before initiation of appropriate highly active antiretroviral therapy.

Department of Microbiology, Queen Mary Hospital, The University of Hong Kong, Pokfulam, Hong Kong
JHK Chen, BSc, PhD
SWC To, MBiochem
WC Yam, PhD, FRCPath
Integrated Treatment Centre, Special Preventive Programme, Centre of Health Protection, Department of Health, Hong Kong
KH Wong, MB, BS, FHKAM (Medicine)
KKC Chan, MB, BS, FHKAM (Medicine)
Department of Medicine, Queen Elizabeth Hospital, 30 Gascoigne Road, Hong Kong
PCK Li, MB, BS, FHKAM (Medicine)
MP Lee, MB, BS, FHKAM (Medicine)

Correspondence to: Dr WC Yam
Email: wcyam@hkucc.hku.hk

Introduction

The use of highly active antiretroviral therapy (HAART) has proven remarkably effective in controlling the progression of human immunodeficiency virus (HIV) disease and thus prolonging survival.[1] However, these benefits can be compromised by the development of antiretroviral drug resistance.[2,3]

There are currently more than 60 International AIDS Society (IAS)–defined antiretroviral drug resistance–related mutations (DRMs), which are associated with resistance to drugs belonging to the six US Food and Drug Administration (FDA)–approved antiretroviral classes.[4] Genotypic resistance tests (GRTs) have been widely used as routine services for HIV-1 drug resistance monitoring in the developed countries.[5] Due to the high

cost of using the FDA-approved commercial HIV-1 genotyping system, resource-limited developing countries may not be inclined to provide this form of testing as a routine.

By means of this study, we tried to study the frequency of DRMs in HIV-1 genotypes that circulate in Hong Kong, using a cost-effective in-house HIV-1 GRT suitable for use with both subtype B and non-B HIV-1 variants.

使用一套耐藥基因型的內部測試系統找出香港人類免疫缺陷病毒一型（HIV-1）高效抗逆轉病毒治療（HAART）耐藥基因變異的情況

目的 使用HIV-1基因型耐藥檢測的內部測試系統找出香港HIV-1 *pol*基因中HAART耐藥基因變異的情況。

設計 回顧研究。

安排 香港兩間HIV診所。

患者 使用一個改良了的基因型耐藥檢測的內部測試系統，把965名HIV患者中的1165個血漿樣本作*pol*基因序列分析。得出結果與美國食品藥品監督管理局（FDA）認可的基因變異測試系統（ViroSeq HIV-1 genotyping system）作比較。最後把測試結果呈報美國斯坦福大學HIV-1耐藥數據庫作分析用途。

結果 我們所使用的病毒基因型耐藥內部測試系統價值40美元，其表現可媲美FDA認可的基因變異測試系統（ViroSeq）。這內部測試系統對於本港最常見的兩種HIV-1亞型毒株（HIV-1 subtype B and CRF01_AE）的檢測上限可達至每毫升400拷貝。耐藥變異只在治療失敗的病人經治療後的血漿樣本中檢測得到。至於兩種HIV-1亞型毒株（subtype B and CRF01_AE）的耐藥變異頻率則無顯著分別。

結論 對於兩種HIV-1亞型毒株（subtype B and CRF01_AE）耐藥基因變異的頻率，經過高成本效益的病毒基因型耐藥內部系統測試，發現並無顯著分別。香港必須要發展一套針對不同HIV-1基因型的數據庫，以加強對於HIV病毒治療的指引。

Methods

Sample collection

Between 2005 and 2009, 1165 plasma samples from 965 HIV-infected patients were collected in two HIV clinics in Hong Kong. Single pre-treatment samples were available from 865 treatment-naïve patients, whereas paired pre-treatment and post-treatment samples were available from 20 virologic treatment-responsive and 80 virologic treatment-failure patients. Post-treatment samples were collected from 12 to 24 months after starting HAART with either two nucleoside reverse transcriptase inhibitors (NRTIs) plus one protease inhibitor (PI) or two NRTIs plus one non–nucleoside reverse transcriptase inhibitor (NNRTI). Virologic treatment responsiveness was defined as demonstration of a viral load of less than 400 copies/mL within 24 weeks of initiating HAART and without any evidence of immunological or clinical failure. Virologic treatment failure in a patient was defined as the presence of a viral load of more than 1000 copies/mL on more than two occasions with or without associated immunological or clinical failure.

Protease and reverse transcriptase nucleotide sequence determination

Viral RNA was extracted from each of the 500 µL patient plasma samples. The plasma samples were stored in -80°C and viral extraction was performed in batches; the samples were first centrifuged at 20 000×g and 4°C for 90 minutes and then extracted using the QIAamp Viral RNA Extraction Kit (Qiagen, Hilden, Germany). The entire protease (PR) and the first 400 codons of the reverse transcriptase (RT) of all 1165 samples were generated by an in-house genotyping method.[6] The amplification and sequencing primers used in the in-house method had been modified from our previous protocol due to the high polymorphic nature of HIV, which weakened the binding efficiency of the previous primer sets. The modified primers were optimised for HIV-1 variants circulating in the Asia Pacific region (Table 1). Sequences were then generated by the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, CA, US). Among the 1165 samples, 350 treatment-naïve, 20 treatment-responsive and 80 treatment-failure samples were also genotyped using an FDA-approved ViroSeq HIV-1 Genotyping System (version 2.0), as a means of evaluating the performance of the in-house GRT.

TABLE 1. In-house primers used for reverse transcriptase–polymerase chain reaction (RT-PCR), nested PCR and cycle sequencing

Primer name	Sequence (5' > 3')	HXB2 position
First RT-PCR		
HIVGRT1F	GCAAGRTTTTGGCBGARGCAATGAG	1867>1892
HIVGRT1R	GACATTTATCACAGYWGGCTACTATTT	4359<4333
Nested PCR		
HIVGRT2F	GGAAAAAGGGCTGTTGGAAATGTG	2015>2038
HIVGRT2R	TTAGTYTCCCTRYTAGCTGCCCCATC	3901<3876
Sequencing		
HIVSEQF2	GCCTGAAAAYCCATAYAATACTCCA	2702>2726
HIVSEQF4	GAGAGACAGGCTAATTTTTTAGG	2071>2093
HIVSEQF5	ACACCTGCCAACATAATTGGA	2490>2510
HIVSEQR1	GGAGGGGTATTRACAAAYTCCCA	3811<3789
HIVSEQR2	TGGATTTTKTDHTCTAAARGGCTCT	3173<3099
HIVSEQR3	GGTACAGTGTCAATAGGACTAATTGGGAA	2575<2547
HIVSEQR4	CCATTCCTGGCTTTAATTTTACTG	2598<2575

Quality assurance of the in-house genotyping test

The sensitivity and specificity of the in-house GRT were evaluated through participation in the TREAT Asia Quality Assurance Scheme (TAQAS) held by the National Serology Laboratory, Australia.[7] A total of five testing panels with 25 proficiency testing samples were genotyped using the in-house system. They were clinical samples of different HIV-1 genotypes, collected from patients in different parts of the Asia-Pacific region. The sequences were then compared with the reference results provided by the organiser. The detection limit of the in-house assay on subtype B and CRF01_AE was investigated using plasma samples with serial dilutions as follows: 20 000, 10 000, 2000, 1000, 400, 200, 100 copies/mL.

TABLE 2. Frequency of the major drug resistance mutations in samples obtained from HIV-1 subtype B and CRF01_AE treatment-failure patients

Mutation	HIV-1 subtype*		P value†
	CRF01_AE	B	
Reverse transcriptase			
Nucleoside reverse transcriptase inhibitors			
M41I/L	6/37 (16.2)	7/28 (25.0)	0.54
D67N	11/37 (29.7)	9/28 (32.1)	1.0
K70G/R	5/37 (13.5)	5/28 (17.9)	0.73
L210W	3/37 (8.1)	4/28 (14.3)	0.45
T215C/E/F/I/S/Y	8/37 (21.6)	11/28 (39.3)	0.17
K219E/N/Q	7/37 (18.9)	6/28 (21.4)	1.0
K65R	1/6 (16.7)	1/7 (14.3)	1.0
L74V	5/9 (55.6)	2/7 (28.6)	0.36
Q151M	1/9 (11.1)	1/7 (14.3)	1.0
M184I/V	34/39 (87.2)	25/29 (86.2)	1.0
Non-nucleoside reverse transcriptase inhibitors			
K103N	4/15 (26.7)	12/15 (80.0)	0.0092
V106A/M	1/15 (6.7)	0/15 (0)	1.0
Y181C/I	5/15 (33.3)	4/15 (26.7)	1.0
G190A/E/S	5/15 (33.3)	2/15 (13.3)	0.39
Entire protease			
L23I	0/26 (0)	1/24 (4.2)	0.48
D30N	0/26 (0)	5/24 (20.8)	0.0201
V32I	1/26 (3.8)	1/24 (4.2)	1.0
L33F	1/26 (3.8)	3/24 (12.5)	0.34
M46I/L	11/26 (42.3)	8/24 (33.3)	0.57
I47A/V	3/26 (11.5)	1/24 (4.2)	0.61
G48Q	0/26 (0)	1/24 (4.2)	0.48
I50L/V	1/26 (3.8)	0/24 (0)	1.0
I54A/L/M/V	5/26 (19.2)	5/24 (20.8)	1.0
L76V	2/26 (7.7)	0.24 (0)	0.49
V82A/F/T	3/26 (11.5)	4/24 (16.7)	0.70
I84V	5/26 (19.2)	1/24 (4.2)	0.19
N88D/S	3/26 (11.5)	4/24 (16.7)	0.70
L90M	4/26 (15.4)	7/24 (29.2)	0.31

* Data are shown in No. of samples out of the total No. of patients receiving corresponding antiretroviral drug in the group, with % in brackets

† P values were calculated with Fisher's exact test

Drug resistance analysis

The individual sequence fragments generated by the in-house system were aligned and manually edited with the Lasergene version 8.1 system (DNASTAR, Wisconsin, US). For the ViroSeq system–generated sequences, the ViroSeq HIV-1 Genotyping Software was used for sequence editing and alignment. A phylogenetic tree was plotted with the edited sequences, so as to prevent cross-contamination during amplification or DNA sequencing. All the edited sequences were submitted to the REGA Genotyping Tool version 2.0 for HIV genotyping and the Stanford HIVdb database (http://hivdb.stanford.edu) for drug resistance interpretation.[8,9] The susceptibilities of 20 antiretroviral drugs were interpreted. They included: zidovudine (AZT), lamivudine (3TC), didanosine (ddI), stavudine (d4T), abacavir (ABC), emtricitabine (FTC), tenofovir (TDF), delavirdine (DLV), efavirenz (EFV), etravirine (ETR), nevirapine (NVP), atazanavir (ATV), darunavir (DRV), fosamprenavir (FPV), indinavir (IDV), lopinavir (LPV), ritonavir (RTV), nelfinavir (NFV), saquinavir (SQV) and tipranavir (TPV).

Results

Quality assurance of the in-house genotypic resistance test

With the five TAQAS HIV-1 proficiency panels, the in-house GRT–generated results consistently demonstrated over 95% concordance with the reference sequence results. The TAQAS panels included samples with multiple HIV-1 genotypes (subtype B, C and CRF01_AE), some of which carried DRMs in the PR/RT region.

Different aspects of the in-house and ViroSeq HIV-1 genotyping systems were compared in this study. Both systems required 2 to 3 days of processing time and similar equipment was used for processing. With respect to all 450 clinical samples, complete concordance in the DRM patterns was identified between the two systems. By genotyping the serially diluted HIV-1–positive plasma samples, the in-house GRT successfully amplified samples with viral loads down to 400 copies/mL on both clinical subtype B and CRF01_AE HIV-1 samples, which demonstrated comparable results to the FDA-approved ViroSeq genotyping system (recommended for samples with viral loads of >1000 copies/mL). However, the reagent cost for running the in-house system was around

US$40 per sample, which was only one quarter the cost of using the ViroSeq system (US$160).

Subtype distribution in Hong Kong

In this study, viral sequences of all 1165 samples were successfully sequenced by the in-house GRT. Among the 965 patients, 444 (46%) were infected with subtype B virus and 415 (43%) with the CRF01_AE virus. Another 99 patients were infected with other defined genotypes including subtype A1 (4 patients; 0.4%), C (40 patients; 4.1%), D (2 patients; 0.2%), G (3 patients; 0.3%), CRF02_AG (7 patients; 0.7%), CRF07_BC (7 patients; 0.7%), CRF08_BC (6 patients; 0.6%), CRF33_01B (2 patients; 0.2%), and other unique recombinant forms (35 patients; 3.6%).

Frequency of major drug resistance mutations

The GRT results from the samples of all treatment-naïve and treatment-responsive patients revealed no IAS-defined major DRM in the viral *pol* gene.

Regarding the 80 treatment-failure patients, they received NRTIs in their initial HAART regimen and at least one IAS-defined NRTI resistance–related mutation was identified in the sequences of their post-treatment samples. The frequency of the major DRMs for NRTIs, NNRTIs, and PIs among the post-treatment samples of subtype B and CRF01_AE samples are presented in Table 2. In the RT region, thymidine analogue mutations (TAMs) which could decrease the susceptibility to AZT, d4T, ddI, ABC and TDF were identified in 43% (31/72) of the patients who had received AZT or d4T. The TAMs were generally more prevalent in patients with HIV-1 subtype B (50%; 14/28) compared to the CRF01_AE subtype (35%; 13/37), but this difference was not statistically significant (P=0.17-1.0). Among the different TAMs, T215C/E/F/I/S/Y was the most common. This mutation was also found in every sample that has developed the M41I/L mutation.

Regarding those who had received 3TC in their initial HAART regimen, a high proportion of subtype B (86%; 25/29) and CRF01_AE (87%; 34/39) patient samples showed the M184I/V mutation that causes high-level 3TC resistance.

Among the 80 treatment-failure patients, 33 (41%) including 15 subtype B, 15 CRF01_AE and 3 other subtypes had exposure to NNRTI in their initial HAART regimen. A primary NNRTI resistance mutation K103N was identified in 16 of these 33 NNRTI-treated patients (B: 80% [12/15]; CRF01_AE: 27% [4/15]; others: 67% [2/3]). In subtype B viruses, K103N development was significantly greater than in CRF01_AE viruses (P=0.0092). Other primary NNRTI resistance mutations including V106A/M, Y181C/I and G190A/E/S were occasionally found in subtype B– and CRF01_AE–infected patients, who had received NNRTIs.

Regarding another 47 patients who had received PIs in their HAART regimen, 27 (57%) of them carried viruses which had developed at least one major PI resistance mutation (B: 50% [12/24]; CRF01_AE: 54% [14/26]; others: 33% [1/3]). Most of the primary PI resistance mutations showed no significant difference between subtype B and in CRF01_AE viruses, except D30N. This NFV-induced major PI resistance mutation was identified in 21% of subtype B patients but not in any patient carrying HIV-1 with other genotypes (P=0.0201). The M46I/L mutation was the most commonly found major PI resistance mutation in this study. It was observed in 33% (8/24) of subtype B–infected and 42% (11/26) of CRF01_AE–infected patients who had exposure to IDV, LPV, NFV, RTV or SQV. The L90M mutation was also observed in 29% (7/24) of subtype B– and 15% (4/26) of CRF01_AE–infected patients, who had received NFV or IDV treatment. Other major PI resistance mutations including L23I, V32I, L33F, I47A/V, G48Q, I50L/V, I54A/L/M/V, L76V, V82A/F/T, I84V and N88D/S were also observed in the sequences of the subtype B and CRF01_AE samples.

Discussion

Resorting to GRT has become an essential tool for HIV-1 drug resistance monitoring. Although the FDA-approved genotyping has demonstrated high-quality performance on both subtype B and non-B HIV-1 strains, the high running costs of their assays hinder their routine application in developing countries.[6]

This study introduced a modified in-house GRT, which had improved primer binding quality suitable for testing the increased degree of HIV-1 genetic diversity found in our locality.[10] Its performance in the five TAQAS proficiency testing panels also indicated its high sensitivity for identifying multiple HIV-1 genotypes.

Owing to the low running cost of our in-house GRT (US$40) and comparable performance to the commercial FDA-approved ViroSeq HIV-1 genotyping system, it could be widely applied, especially in developing countries.

Through the use of our in-house GRT in genotyping 1165 clinical samples in Hong Kong, we also revealed that the HIV-1 subtype B and CRF01_AE were the predominant genotypes in Hong Kong. Our previous report of a cohort collected between 2000 and 2004 yielded only subtype B, C and CRF01_AE viruses.[11] However, the cohort in this study was collected from 2005 to 2009, and included a greater variety of non-B subtypes and HIV-1 new recombinants. The current study is also consistent with the increasing genetic

diversity of HIV-1 previously reported in Hong Kong.[10]

The absence of resistance mutations in all treatment-naïve and pre-treatment patient samples also demonstrates the low prevalence of primary resistance among HIV-1 patients in Hong Kong.[12] Evidently, all drug resistance mutations developed after starting HAART.

The difference in the frequency of DRMs in HIV-1 subtype B and non-B viruses has been a debated topic for several years.[11,13-17] Generally, most of the DRMs exhibit no significant difference in frequency in subtypes B and CRF01_AE viruses. Our study, however, demonstrated that when exposed to NRTI, the HIV-1 subtype B apparently had a higher chance of developing TAMs compared to HIV-1 CRF01_AE. In addition, a significantly higher rate of K103N development was observed in the former subtype.

In conclusion, our cost-effective in-house GRT is suitable for the detection of low viral load and high genetic diversity HIV-1 samples circulating among developing countries in Asia. Although there is no significant difference in DRM frequencies between subtype B and CRF01_AE, a DRM database for different HIV-1 genotypes should be established as guide for management of HIV infection in our region.

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