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<td><strong>Author(s)</strong></td>
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Increased Genetic Diversity of HIV-1 Circulating in Hong Kong

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Abstract
HIV-1 group M strains are characterized into 9 pure subtypes and 48 circulating recombinant forms (CRFs). Recent studies have identified the presence of new HIV-1 recombinants in Hong Kong and their complexity continues to increase. This study aims to characterize the HIV-1 genetic diversity in Hong Kong. Phylogenetic analyses were performed by using HIV-1 pol sequences including protease and partial reverse transcriptase isolated from 1045 local patients in Hong Kong from 2003 to 2008. For the pol sequences with unassigned genotype, the evidence of recombination was determined by using sliding-window based bootscan plots and their env C2V3 region were also sequenced. Epidemiological background of these patients was further collected. The pol phylogenetic analyses highlighted the extent of HIV-1 genetic diversity in Hong Kong. Subtype B (450/1045; 43.1%) and CRF01_AE (469/1045; 44.9%) variants were clearly predominant. Other genotypes (126/1045; 12.1%) including 3 defined subtypes, 10 CRFs, 1 unassigned subtype and 33 recombinants with 11 different mosaic patterns were observed. Recombinants of subtype B and CRF01_AE were mainly found among local Chinese MSM throughout 2004 to 2008, while the CRF02_AG and subtype G recombinants were circulating among non-Chinese Asian population in Hong Kong through heterosexual transmission starting from 2008. Our study demonstrated the complex recombination of HIV-1 in Hong Kong and the need in developing surveillance system for tracking the distribution of new HIV-1 genetic variants.

Introduction
Human immunodeficiency virus (HIV) carries an error prone reverse transcriptase which causes its extraordinary genetic diversity [1]. Throughout the epidemic, 9 subtypes (A–D, F–H, J and K) and a further 48 major circulating recombinant forms (CRFs) has been documented within HIV type 1 (HIV-1) M group (http://www.hiv.lanl.gov/content/hiv-db/CRFs/CRFs.html). Genetic founder effect causes the heterogeneous and specific global geographic distribution of different HIV-1 group M strains [2]. HIV-1 subtype C infection is predominant in the world while the epidemic in the western world is primarily caused by HIV-1 subtype B variants [3]. In the East and Southeast Asia, CRF01_AE is highly predominant.

Meanwhile, the frequent self-recombination and ongoing exchange of HIV-1 strains between geographic regions through population migration and travel caused a small number of group M strains to move into new host populations [2]. In recent years, growing numbers of HIV-1 recombinant forms have been identified in different geographical regions [2]. The CRF01_AE, CRF07_BC, CRF08_BC, CRF33_01B and CRF34_01B were confirmed to be originated in Asia while new unique recombinant forms (URFs) are continually being identified in these years and their complexity continues to increase [4,5,6]. These URFs may later become new CRFs after circulating in particular geographical areas.

Up to the end of 2006, the HIV-1 subtype B (36.4%) and CRF01_AE (48.8%) are co-predominant in Hong Kong [7,8]. Through phylogenetic estimation, the divergence date of these strains in Hong Kong is around 1995–2001 [9,10]. However, the prevalence of other new recombinant forms in Hong Kong remains unclear. Hong Kong is a metropolitan city located at the centre of the Southeast Asia. The high migration and traveling population of Hong Kong with other HIV-1 epidemic Asian countries such as Mainland China, Thailand, India, Malaysia, etc. may enhance the development of new HIV-1 recombinants in the Southeast Asia.

As the rising number of new recombinant lineages can significantly impact therapeutics and vaccine development [11,12], this study aims to investigate the prevalence and characterization of new recombinants circulating in Hong Kong from 2003 to 2008. Also, we would like to track the epidemiological transmission reservoir of these recombination variants.

Results
Distribution of HIV-1 group M variants in Hong Kong
The 1045 individuals were recruited in the Integrated Treatment Centre of the Department of Health from January 2003 to December 2008 which represented 51.4% (1045/2032) of all new diagnoses of HIV-1 infection in Hong Kong within that...
phylogenetic analysis could assign confident genotypes to 96.3% of
samples (1006/1045), while subtype B and CRF01_AE accounted
for 43.1% (450/1045) and 44.9% (469/1045) of the total number of
samples respectively. HIV-1 variants with other genotypes were
recognized in 12.1% of samples (126/1045) including subtype A1
(1/126; 0.8%), C (44/126; 34.9%), D (1/126; 0.8%), CRF02_AG
(7/126; 5.6%), CRF03_AB (2/126; 1.6%), CRF06_cpx (1/126;
0.8%), CRF07_BC (20/126; 15.9%), CRF08_BC (9/126; 7.1%),
CRF15_01B (2/126; 1.6%), CRF22_01A1 (1/126; 0.8%), CRF33_01B
(2/126; 0.8%), CRF42_02G (1/126; 0.8%) and CRF43_02G
(1/126; 0.8%). There were another 34 samples (34/126; 27.0%)
that could not be confidently assigned to previously defined subtypes
or CRFs by the pol phylogenetic analysis (Figure 1a).

The env C2V3 region of the 34 unassigned samples were further
sequenced and confidently assigned genotypes (bootstrap value
>70) could only be found on 31 samples in the env phylogenetic
tree (Figure 1b) and 3 samples were failed in the env gene PCR-
sequencing. There was no genetic recombination found in any of
the 31 available env sequences while 1 sample was genotyped as
subtype F2-like outgroup by using pol gene and CRF02_AG by
using env gene (Table 1).

Through the bootscanning recombination analysis, 33 out of the
34 unassigned samples were found to have genetic recombination
in the pol gene and another one was classified as unassigned
subtype F2 (U^F2). The bootscanning recombination patterns shown in Figure 2. Samples with subtype B and CRF01_AE
recombination in the pol gene were identified in 16 samples with
different recombination patterns (U^B/CRF01/20, U^B/CRF01/2B
and U^CRF01/CRF02/2). Recombination of subtype G and CRF02_AG in the
pol gene (U^G/CRF02) were identified in another 5 samples. Three
samples were found to have subtype B and subtype C
recombination in the pol region with 2 different recombination
patterns (U^B/C and U^C/B), while another 3 samples were
CRF01_AE and subtype C recombinants (UCRF01/C). Recombi-
nation of CRF02_AG and subtype K (UCRF02/K) (2 samples),
CRF02_AG and CRF01_AE (UCRF02/CRF01 and UCRF01/CRF02)
(2 samples), and CRF03_AB and A1 (UCRF03/3A) (2 samples) were
also observed.

Characteristics of new recombinant infected patients

The first recombinant sample in this study was collected in 2004
and the number of cases increased progressively in the following
years (Table 2).

The U^B/CRF01 recombinant was the only recombi-
nation variant that was found every year between 2004 and 2008, while
almost all these samples were collected from Hong Kong Chinese
male residents (13 out of 14). The transmission route among these
Chinese males was found to be MSM (9 samples) and heterosexual
(3 samples) transmission. Another sample was isolated from a male
Vietnamese immigrant with intravenous drug use. For the U^CRF01/B
recombinant, it was isolated from a Chinese heterosexual male and a Filippino female. The Filippino female was confirmed catching disease through heterosexual
transmission.

Another 5 U^G/CRF02 recombinant cases were all isolated in
2008. This recombinant variant infected 2 non-Chinese male
patients including 1 African from Ghana and 1 Bangladeshi. The
other 3 patients were non-Chinese Asian females from Indonesia
(1 patient) and the Philippines (2 patients). All 5 patients arrived in
Hong Kong since 2006 with HIV infection through heterosexual
transmission between 2007 and 2008.

Recombinant of subtype B and C (U^B/C) and U^C/B
were isolated from 3 Chinese males. The U^C/B infected patient was
confirmed catching HIV-1 away from Hong Kong through heterosexual
transmission.

The U^CRF01/C recombinants were identified in 3 heterosexual
patients including 1 Chinese female, 1 Nepalese male and 1
Nepalese female. The two Nepalese were husband and wife while
the wife had been confirmed to catch disease from the male
patient.

For the U^CRF02/K recombinants, cases were reported in 2007
and 2008 which samples were collected from 1 Nigerian male and
1 Filippino female.

The U^CRF03/A1 recombinant was identified in another 2
patients who got infection through heterosexual transmission (1
Chinese male and 1 British Caucasian male) while the U^CRF02 /
CRF01 recombinant was identified in one Nigerian female and the
U^CRF02/CRF01 recombinant was found in one Chinese male.

Discussion

Prior HIV-1 molecular epidemiology studies performed in Asia
were mainly focused on the transmission of locally predominant
HIV-1 genotypes [7,10,13,14]. There was limited number of
studies which focused on new HIV-1 group M recombinant
transmission. In this study, we demonstrated the broad HIV-1
genetic diversity in Hong Kong and it is the first comprehensive
study in Asia revealing the transmission of different new recombinants in the same locality.

Table 1. The pol and env genotype of the 34 unassigned
genotype samples.

<table>
<thead>
<tr>
<th>pol</th>
<th>n</th>
<th>env</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>U^B/CRF01</td>
<td>14</td>
<td>B</td>
<td>9</td>
</tr>
<tr>
<td>CRF01_AE</td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>U^G/CRF02</td>
<td>5</td>
<td>G</td>
<td>5</td>
</tr>
<tr>
<td>U^CRF01/C</td>
<td>3</td>
<td>CRF01_AE</td>
<td>2</td>
</tr>
<tr>
<td>U^B/C</td>
<td>2</td>
<td>B</td>
<td>2</td>
</tr>
<tr>
<td>U^CRF02/CRF01</td>
<td>1</td>
<td>N/A</td>
<td>1</td>
</tr>
<tr>
<td>CRF02_AG</td>
<td>1</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>U^CRF01/CRF02</td>
<td>2</td>
<td>CRF02_AG</td>
<td>2</td>
</tr>
<tr>
<td>U^CRF02/CRF01</td>
<td>2</td>
<td>CRF01_AE</td>
<td>1</td>
</tr>
<tr>
<td>U^CRF02/X1</td>
<td>2</td>
<td>A1</td>
<td>2</td>
</tr>
<tr>
<td>U^CRF01/CRF02</td>
<td>2</td>
<td>CRF02_AG</td>
<td>1</td>
</tr>
<tr>
<td>U^CRF01/CRF02/2</td>
<td>2</td>
<td>A1</td>
<td>2</td>
</tr>
<tr>
<td>U^CRF02/CRF01</td>
<td>2</td>
<td>CRF02_AG</td>
<td>1</td>
</tr>
<tr>
<td>CRF02_AG</td>
<td>1</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>CRF08_BC</td>
<td>1</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>CRF02_AG</td>
<td>1</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>34</td>
<td></td>
<td>34</td>
</tr>
</tbody>
</table>

N/A represents PCR amplification or DNA sequencing failed.
doi:10.1371/journal.pone.0012198.t001
The treatment naïve HIV-1 samples in this study were collected from 2003 to 2008 in Hong Kong and the study cohort herein was estimated to represent about 50% of the total HIV-1 patients in Hong Kong within the 5 years [15]. Among the total HIV-1 patients in Hong Kong, about 60–70% of them were local Chinese inhabitants, while the other 30–40% of patients was non-Chinese residents including non-Chinese Asians, Caucasians and Africans. From the large sample size included in this study, we can further confirm that subtype B (45.1%) and CRF01_AE (44.9%) were co-dominant HIV-1 genotypes among the local Chinese patients in Hong Kong. This genotyping distribution showed complete concordance to our recent studies [7,10]. Other than these two predominant genotypes, another 12 defined subtypes or CRFs were also identified, while subtype C and CRF07_BC were more commonly found. Other defined HIV-1 CRFs, such as CRF22_01A1, CRF12_02G and CRF43_02G were more sporadically found in Hong Kong and these CRFs are first ever reported in China and Hong Kong. This broad range of defined HIV-1 genotypes suggested the multiple route of transmission for the local Chinese Asian and African who arrived to Hong Kong starting from 2006. They were estimated to catch HIV-1 infection through heterosexual transmission between 2007 and 2008. As subtype G and CRF02_AG were not commonly found in Hong Kong in the past 10 years, we believe this UG/CRF02 recombinant is not originated from the local circulating subtype G and CRF02_AG strains. Since the viral pol sequences of the 5 UG/CRF02 cases demonstrated a 98% similarity, it is plausible that there might be linkages between the sources of infection with this recombinant strain in these non-Chinese cases. Although this study could not prove the origin of this new UG/CRF02 recombinant, there is high probability that the UG/CRF02 recombinant was imported to Hong Kong between 2007 and 2008 through non-Chinese HIV-1 carriers.

Table 2. The epidemiological background of the 33 genotype unassigned recombinants.

<table>
<thead>
<tr>
<th>Category</th>
<th>pol gene recombination</th>
<th>Number of samples</th>
<th>Ethnicity and gender (transmission route)</th>
<th>Sampling year</th>
</tr>
</thead>
<tbody>
<tr>
<td>UB/CRF01(b)</td>
<td>B/CRF01_AE</td>
<td>4</td>
<td>4 Chinese males (3 MSM and 1 heterosexual)</td>
<td>2005, 2007</td>
</tr>
<tr>
<td>UG/CRF02</td>
<td>G/CRF02_AG</td>
<td>5</td>
<td>2 Filipino females (heterosexual)</td>
<td>2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 Indonesian female (heterosexual)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 Bangladeshi male (heterosexual)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 Ghanaian male (heterosexual)</td>
<td></td>
</tr>
<tr>
<td>UB/CRF01/C</td>
<td>CRF01_AE/C</td>
<td>3</td>
<td>1 Chinese female (heterosexual)</td>
<td>2006, 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 Nepalese male (heterosexual)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 Nepalese female (heterosexual)</td>
<td></td>
</tr>
<tr>
<td>UC/CRF01/A</td>
<td>CRF01_AE/B</td>
<td>2</td>
<td>1 Chinese male (heterosexual)</td>
<td>2006, 2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 Filipino female (heterosexual)</td>
<td></td>
</tr>
<tr>
<td>UC/CRF01/K</td>
<td>B/C</td>
<td>2</td>
<td>1 Chinese male (heterosexual)</td>
<td>2004, 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 Filipino female (heterosexual)</td>
<td></td>
</tr>
<tr>
<td>UC/CRF02/K</td>
<td>CRF02_AG/K</td>
<td>2</td>
<td>1 Nigerian male (heterosexual)</td>
<td>2007, 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 Filipino female (heterosexual)</td>
<td></td>
</tr>
<tr>
<td>UC/CRF03/A1</td>
<td>CRF03_AB/A1</td>
<td>2</td>
<td>1 Chinese male (heterosexual)</td>
<td>2006, 2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 British male (heterosexual)</td>
<td></td>
</tr>
<tr>
<td>UC/CRF01/CRF02</td>
<td>CRF01_AE/CRF02_AG</td>
<td>1</td>
<td>1 Chinese male (heterosexual)</td>
<td>2004</td>
</tr>
<tr>
<td>UC/CRF02/CRF01</td>
<td>CRF02_AG/CRF01_AE</td>
<td>1</td>
<td>1 Nigerian male (heterosexual)</td>
<td>2005</td>
</tr>
<tr>
<td>UC</td>
<td>C/B</td>
<td>1</td>
<td>1 Chinese male (heterosexual)</td>
<td>2004</td>
</tr>
</tbody>
</table>
In conclusion, our study revealed the broad genetic diversity of HIV-1 in Hong Kong and identified two local spreading new recombinant lineages. Since the rising number of new recombinant lineages can significantly impact therapeutics and vaccine development, therefore, a good surveillance system for tracking the distribution of new HIV-1 genetic variants will be necessary.

Materials and Methods

Ethics approval has been obtained from the Institutional Review Board of the University of Hong Kong/Hospital Authority (Hong Kong West Cluster) with Reference number UW08-070.

Patient samples

This study included the first available plasma samples isolated from 1045 HIV-1 positive patients who had used the genotyping resistance testing service of the Department of Health between 2003 and 2008. All patient samples were reported to be treatment naive at the moment of collection.

HIV sequencing and phylogenetic analysis

The HIV-1 pol sequences were amplified and sequenced by ViroSeq HIV-1 Genotyping System version 2.0 (Celera Diagnostics, CA) or an in-house genotyping method as described previously [8]. All the pol sequences incorporated entire protease (297 base pairs; 99 codons) and partial reverse transcriptase (828 base pairs; 276 codons) region, with a total length of 1125 base pairs. The HIV-1 genotypes were then determined by the phylogenetic analysis. The pol sequences were aligned with HIV-1 group M 2009 reference sequences in the NCBI Viral Genotyping Tool (http://www.ncbi.nlm.nih.gov/projects/genotyping) by using MUSCLE [16]. Phylogenetic tree was constructed with PAUP* 4.0 using the neighbor-joining (NJ) algorithms with 1000 bootstrap replicates [17]. In order to determine the clade assignments and recombinant structure of the genotype unassigned sequences, bootscanning analyses were performed on the genotype unassigned samples with the SimPlot software [18]. The SimPlot performed bootscanning on neighbor-joining trees by using SEQBOOT, DNAVIST, NEIGHBOR and CONSENSE from the PHYLIP package on a moving window of 300–400 base pairs along the alignment with 50 base pairs increments. The bootstrap values for the studied sequences were plotted at the midpoint of each window. In the analysis, the new sequences were compared with consensus sequences (50% threshold) representing the HIV-1 variants from the same alignment used for phylogenetic tree analysis.

Furthermore, the env C2V3 region of the unassigned samples were amplified and sequenced by 2 pairs of in-house primers (ENV1F 5′-TAGGGATCTCGTATGAGCGAGAAGGCG3′; ENV1R 5′-CAGCTTCTCAATGTCYTTATATYTTCC-TCC1CCAGG-3′; ENV2F 5′-ATACATTATTGCGCYCRGCTGG3′ and ENV2R 5′-ATGGAAGGCCCATAYATTGCG-3′). The 480 base pair sequences were then aligned with env sequences of the NCBI HIV-1 2009 reference set.

Epidemiology data analysis

For determining the epidemiological transmission reservoir of the unassigned genotype samples, the patient epidemiological information including the age, gender, ethnicity, place of birth, route of transmission, plasma sampling date were collected from the Integrated Treatment Centre, Department of Health.

Acknowledgments

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Author Contributions

Conceived and designed the experiments: JC WCY. Performed the experiments: JC HYL SWCT. Analyzed the data: JC KHW ZC HYL WCY. Wrote the paper: JC.