Utilization of a Duplex HybProbe Real-Time PCR to Detect and Estimate IL-28B Polymorphisms Prevalence among HIV/HCV Co-infected Patients in Hong Kong

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Abstract

Conventional treatment for chronic HCV infection relies on the combination of peg-interferon and ribavirin therapy. Both interleukin-28B (IL-28B) polymorphisms and HCV genotypes serve as the strongest predictive values for therapeutic prognosis. The treatment regimens for HIV/HCV co-infected patients are more complex and dependent on various host immune and viral factors. A rapid and cost-effective IL-28B genotyping tool is therefore crucial to assist clinicians on better patient management. This study aimed to evaluate the performance of a newly developed HybProbe duplex real-time PCR assay in detecting IL-28B polymorphisms among HIV/HCV co-infected patients in Hong Kong. A total of 88 HIV/HCV co-infected patients were recruited at the Integrated Treatment Centre during 2009 to 2014. IL-28B polymorphisms on rs12979860 and rs8099917 were determined by an in-house HybProbe assay with melting curve analysis. For assay evaluation, IL-28B polymorphisms of 46 samples with diverse HIV/HCV genotypes were confirmed by Sanger sequencing. Both in-house HybProbe assay and sequencing results for IL28B polymorphisms determination were completely concordant. Among the 88 HIV/HCV co-infected, the frequency of rs12979860 wild-type (C/C) was 88.6%, heterozygous mutant (C/T) was 9.1% and remaining 2.3% homozygous mutant (T/T). The prevalence of IL-28B polymorphisms in rs8099917 was slightly differed, which had 90.9% wild-type (T/T), 6.8% heterozygous mutant (G/T) and 2.3% homozygous mutant (G/G). This novel duplex assay could allow clinicians to make early decision on treatment option for HIV/HCV co-infected patients by detecting rs12979860 and rs8099917 polymorphisms simultaneously.

Background

In developed countries, the morbidity and mortality in Human Immunodeficiency Virus (HIV) patients is significantly induced by chronic Hepatitis C Virus (HCV) co-infection. It is estimated to have about one-third of HIV patients co-infected with HCV. These patients usually require immediate HCV treatment as they tolerate poorly towards antiretroviral treatment, progress more rapidly to end-stage liver disease and often lead to death [1,2]. For decades, the standard therapy for HCV infection relies on the combination of pegylated interferon and ribavirin (PEG-IFN/RBV). This treatment is known to be less effective towards HIV/HCV co-infected patients, with a lower rate of Sustained Virological Response (SVR) and a higher rate of viral relapse upon treatment completion [3,4]. Recent studies identified two Single Nucleotide Polymorphisms (SNPs) rs12979860 and rs8099917 that located near interleukin-28B (IL-28B) and acted as important baseline predictors for PEG-IFN/RBV treatment responses. Strong association between favourable genotypes (C/C for rs12979860 and T/T for rs8099917) and high rate of SVR were observed in several genome wide association studies [5-8]. Clinical management on HIV/HCV co-infected patients is always more complicated than mono-infected patients. Several commercial assays and sequencing are the major IL-28B polymorphisms detection methods, yet they are relatively expensive and time-consuming. This study aimed to develop a rapid and cost-effective duplex HybProbe real-time assay to detect both rs12979860 and rs8099917 SNPs in a single reaction and estimate the prevalence of IL-28B, HIV-1 and HCV subtypes among co-infected patients in this locality.

Objectives

To evaluate the performance of a newly developed HybProbe duplex real-time PCR assay in detecting IL-28B polymorphisms on rs12979860 and rs8099917, and to estimate the prevalence of IL-28B polymorphisms among HIV/HCV co-infected patients in Hong Kong.

Study Design

A total of 88 HIV/HCV co-infected patients were recruited at the Hong Kong Government Integrated Treatment Centre during 2009 to 2014. Whole blood samples were collected after patient consent. The IL-28B polymorphisms on rs12979860 and rs8099917 of all samples were detected by the HybProbe assay with melting curve analysis. The duplex PCR assay was amplified by LightCycler® FastStart DNA Master HybProbe (Roche Diagnostics, Germany), with two pairs of primers and hybridization probes specifically targeting rs12979860 and rs8099917 (Table 1). For assay evaluation, IL-28B polymorphisms of 46 samples were further confirmed by Sanger sequencing. HCV viral load was quantitated by Abbott m2000 RealTime HCV assay (Abbott Laboratories, USA) while HIV-1 and HCV genotypes were confirmed by sequencing.

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Results

Basic demographic characteristics of HIV/HCV co-infected patients in this study are presented in Table 2. There were 82 males (93.2%) included in this study, with an overall median age at 39 (range 21 – 63 years). Nearly 65% of the study population were Chinese with approximately 33% non-Chinese Asians. The in-house HIV-1 genotyping analysis revealed that the patients were infected by subtype B (20.5%), CRF01_AE (47.7%) and C or BC recombinants (15.9%). HCV genotypes were detected in 71 patients, including genotype 1/1a/1b (30.7%), 3a/3b (26.1%) and 6a/6d/6e (23.9%). Due to low HIV-1 or HCV viral load at sample collection, about 15 – 20% of the co-infected patients were unable to have either or both viral genotyping results.

The HybProbe assay performance was evaluated among 46 co-infected samples, using Sanger sequencing as gold standard. Diverse HIV-1 subtypes (5 subtype B, 3 subtype C, 29 CRF01_AE, 4 CRF07_BC, 1 CRF08_BC and 4 undetermined) and HCV genotypes (2 genotype 1, 9 genotype 1a, 6 genotype 1b, 1 genotype 3a, 3 genotype 3b, 9 genotype 6a, 1 genotype 6d, 3 genotype 6e and 13 undetermined) samples were included for evaluation. Sanger sequencing required two individual reactions and manual sequence proof-reading whereas in-house HybProbe assay required melting curve analysis to interpret the SNPs on rs12979860 and rs8099917. Rs12979860 C/C wild-type had a melting peak at 64 ± 1°C while T/T mutant had a lower melting temperature at 54 ± 1°C (Figure 1A). For rs8099917, T/T wild-type melted at 64 ± 1°C whereas mutant G/G melted at 57 ± 1°C (Figure 1B). Heterozygous mutants (C/T of rs12979860 and T/G of rs8099917) could be typically interpreted when both melting peaks of wild-type and mutant were seen. Both systems

| rs12979860 | Forward Primer | 5'- GCGCTTATCCTGATACGGCTA - 3' |
| rs12979860 | Reverse Primer | 5'- TCACAGAAAGGAGGACCTGCC - 3' |
| rs12979860 | Sensor Probe | 5’- CGAAGGCGCGAACCAGG-Fluorescein - 3' |
| rs12979860 | Anchor Probe | 5'- LC Red 640 - TGAATTGCTCCGCGCTCCC-Phosphate - 3' |

| rs8099917 | Forward Primer | 5'- AAGTAACACTTGTCCTGTAAAGATTCC - 3' |
| rs8099917 | Reverse Primer | 5'- CGCTATAATTAAGATGTTGGGAGAATGCAA - 3' |
| rs8099917 | Sensor Probe | 5' - CCTTTCTGTGAGCAATTTCACCCAA- Fluorescein - 3' |
| rs8099917 | Anchor Probe | 5' - LC Red 705 - TGGAACCATGCTGTATACTAGTGTGC-Phosphate – 3' |

C or T indicates SNPs position.

Table 1: Primers and probes for IL-28B Genotyping assays.

<table>
<thead>
<tr>
<th>Total</th>
<th>rs12979860</th>
<th>P-value*</th>
<th>rs8099917</th>
<th>P-value*</th>
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<tr>
<td>n (%)</td>
<td>78 CC</td>
<td>10 CT/TT</td>
<td>80 TT</td>
<td>8 TG/GG</td>
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<td>Gender</td>
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<td>73 (83.0)</td>
<td>9 (10.2)</td>
<td>75 (85.2)</td>
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<td>Female 6 (6.8)</td>
<td>5 (5.7)</td>
<td>1 (1.1)</td>
<td>5 (5.7)</td>
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<td>Ethnicity</td>
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<td>50 (56.8)</td>
<td>7 (8.0)</td>
<td>50 (56.8)</td>
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<tr>
<td></td>
<td>Asian 29 (33.0)</td>
<td>27 (30.7)</td>
<td>2 (2.3)</td>
<td>28 (31.8)</td>
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<td></td>
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<td>1 (1.1)</td>
<td>1 (1.1)</td>
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<td>Risk of Infection</td>
<td>IDU 18 (20.5)</td>
<td>17 (19.3)</td>
<td>1 (1.1)</td>
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</tr>
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<td>Homosexual 7 (8.0)</td>
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<td>7 (8.0)</td>
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<tr>
<td></td>
<td>Heterosexual 2 (2.3)</td>
<td>2 (2.3)</td>
<td>--</td>
<td>2 (2.3)</td>
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<tr>
<td></td>
<td>Undetermined 61 (69.3)</td>
<td>52 (59.0)</td>
<td>9 (10.2)</td>
<td>54 (61.4)</td>
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<td>HIV-1 subtypes</td>
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<td>14 (15.9)</td>
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<td>CRF01_AE 42 (47.7)</td>
<td>39 (44.3)</td>
<td>3 (3.4)</td>
<td>40 (45.5)</td>
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<td>C or CRF07_BC or CRF08_BC 14 (15.9)</td>
<td>13 (14.8)</td>
<td>1 (1.1)</td>
<td>13 (14.8)</td>
</tr>
<tr>
<td></td>
<td>PCR -ve 14 (15.9)</td>
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<td>1 (1.1)</td>
<td>13 (14.8)</td>
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<tr>
<td>HCV genotypes</td>
<td>1 / 1a / 1b 27 (30.7)</td>
<td>26 (29.5)</td>
<td>1 (1.1)</td>
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<tr>
<td></td>
<td>3a / 3b 23 (26.1)</td>
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<td>5 (5.7)</td>
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<td>6a / 6d / 6e 21 (23.9)</td>
<td>19 (21.6)</td>
<td>2 (2.3)</td>
<td>20 (22.7)</td>
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<tr>
<td></td>
<td>PCR -ve 17 (19.3)</td>
<td>15 (17.0)</td>
<td>2 (2.3)</td>
<td>15 (17.0)</td>
</tr>
</tbody>
</table>

N/A: Not available; -ve: negative; IDU: Intravenous drug users
*Statistics: Fisher’s exact tests

Table 2: Basic demographic characteristics of HIV/HCV co-infected patients.
Figure 1: Melting curves and melting peaks analysis of rs12979860 (Figure 1A) and rs8099917 (Figure 1B) single nucleotide polymorphisms detection by in-house HybProbe assay. Heterozygous mutants could be interpreted when both melting peaks or wild-type and mutant were seen.
Among the 88 HIV/HCV co-infected patients in this locality, the frequency of rs12979860 wild-type (C/C) was 88.6%, while the frequency of C/T and T/T mutants was 9.1% and 2.3% respectively. The prevalence of IL-28B polymorphisms is rs8099917 was significantly differed (P < 0.0001, Fisher’s exact tests), which had 90.9% wild-type (T/T), 6.8% G/T and 2.3% G/G mutants. This study revealed no association between HIV-1 subtypes, HCV genotypes, ethnicities or gender between rs12979860 or rs8099917 unfavourable genotypes. Most of the patients had concordant wild-type or mutant genotypes between both rs12979860 and rs8099917. Exceptions were seen in one Asian and one patient with undetermined ethnicity as they had unfavourable C/T on rs12979860 but favourable wild-type T/T on rs8099917.

**Discussion**

The identification of both rs12979860 and rs8099917 favourable genotypes largely enhanced the rate of SVR in HIV/HCV co-infected patients. To our knowledge, this is the first in-house duplex IL-28B real-time HybProbe assay that successfully identifies both rs12979860 and rs8099917 SNPs simultaneously in one single tube. The HybProbe assay used LC-RED640 and LC-RED705 acceptor dyes to detect both SNPs in a single run instead of individual sequencing reactions. The melting temperatures for both SNPs interpretation were also very distinct, which was 10°C for rs12979860 and 6°C for rs8099917, respectively. Traditional Sanger sequencing consists of several tedious experimental steps, including end-point PCR and capillary analysis, whereas the in-house duplex assay largely shortened the turnaround time from two days to two hours with at least 50% of running cost reduction.

The in-house HybProbe assay achieved 100% concordant SNPs interpretation with the sequencing results. Both Chinese and Asians were included in this study, together with diverse HIV-1 and HCV genotypes, suggesting that the current assay would be largely applicable to global laboratories. Both HIV-1 subtype B and CRF01-AE accounted for about 80% of local infections in Hong Kong [11]. A higher prevalence of CRF01-AE (47.7%) was noticed in our patients, whereas the prevalence for subtype B was relatively low (20.5%). Limited information on routes of infection was available at the time of study, though about a-fifth of the study population were infected via intravenous drug use. Conversely the prevalence of HCV genotype 1, 3 and 6 were roughly similar, which was inconsistent with previous local studies, suggesting that the HIV/HCV co-infection might predominantly affect intravenous drug users with distinct epidemiological and transmission pattern. [10,12].

Clinical management among HIV/HCV co-infected patients usually induced more complications due to drug-drug interaction and patients’ immune status. Poor SVR rate was mainly associated with HCV genotype 1 and unfavourable IL-28B SNPs yet the information on other HCV genotypes and HIV co-infection was scarce. Two of our patients had C/T mutant on rs12979860 but wild-type T/T on rs8099917. Their treatment response towards 48-week of PEG-IFN/ RBV should be analysed in greater details to assess the impact of discordant IL-28B polymorphisms. Due to data availability, the HIV/HCV treatment history and rate of SVR were insufficient for further investigation on the relationship between IL28B polymorphisms, HIV/HCV genotypes and treatment responses among HIV/HCV co-infected patients.

**Conclusion**

This study developed a simple and efficient IL28B duplex real-time HybProbe assay with excellent performance on rs12979860 and rs8099917 SNPs detection. The rapid detection allows clinicians to make early decision on treatment option and duration. Both C/C and T/T wild-types of rs12979860 and rs8099917 were prevalent in our locality. No significant association were observed between IL28B polymorphisms and HIV/HCV genotypes.

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**References**