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Short Communication Open Access

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 on rs12979860 and rs8099917, and to estimate the prevalence of 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 *m*2000 RealTi*me* HCV assay (Abbott Laboratories, USA) while HIV-1 and HCV genotypes were confirmed

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by our in-house methods [9,10]. Statistical analysis was performed by Graphpad Prism version 6(Graphpad software, California, US).

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, 2 genotype 3a, 1 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°C ± 1°C while T/T mutant had a lower melting temperature at 54°C ± 1°C (Figure 1A). For rs8099917, T/T wild-type melted at 64°C ± 1°C whereas mutant G/G melted at 57°C ± 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'- GCGCTTATCGCATACGGCTA - 3'
Reverse Primer	5'- TCACAGAAGGGAGCCCTGCC - 3'
Sensor Probe	5'- CGAAGGCGCGAACCAGG-Fluorescein - 3'
Anchor Probe	5'- LC Red 640 - TGAATTGCTCCGCGCTCCC-Phosphate - 3'
rs8099917	
Forward Primer	5'- AAGTAACACTTGTTCCTTGTAAAAGATTCC - 3'
Reverse Primer	5'- CGCTATAATTAAAGATGTGGGAGAATGCAA - 3'
Sensor Probe	5' - CCTTTCTGTGAGCAATTTCACCCAA- Fluorescein - 3'
Anchor Probe	5' - LC Red 705 - TGGAACCATGCTGTATACAGTTTGGTAGC-Phosphate – 3'

C or T indicates SNPs position.

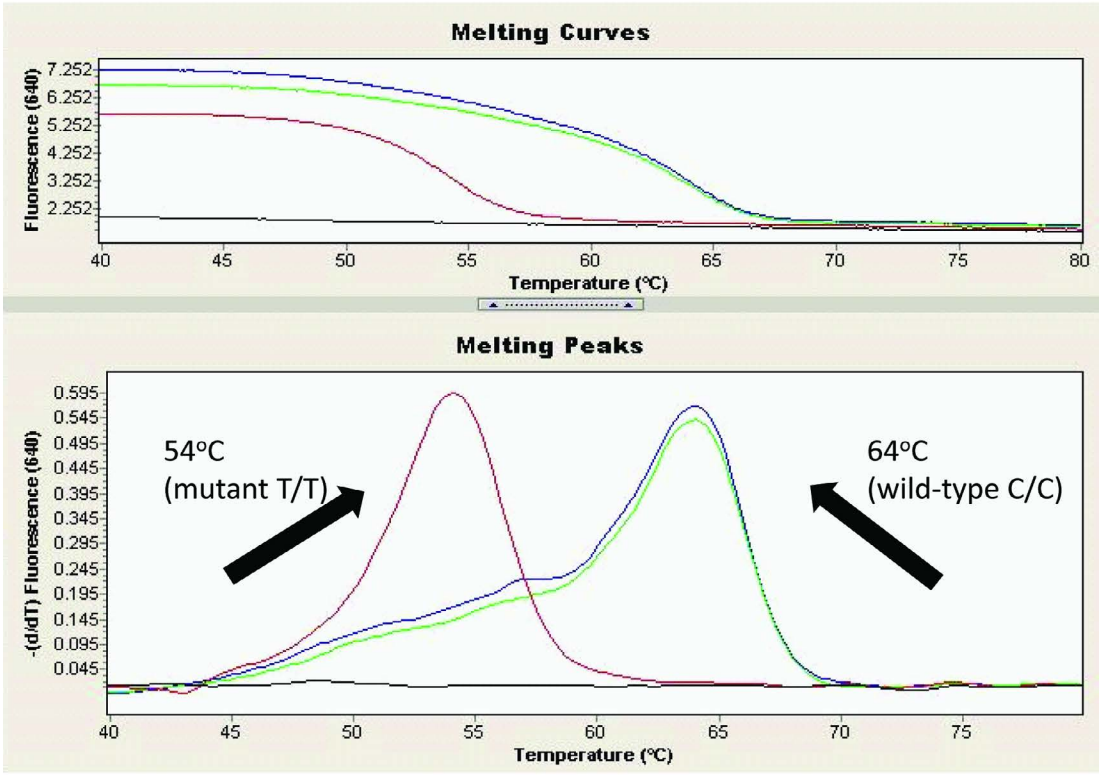
Table 1: Primers and probes for IL-28B Genotyping assays.

	Total	**rs12979860**		***P*-value***	**rs8099917**		***P*-value***
	n (%)	CC	CT/TT		TT	TG/GG	
		(n=78)	(n=10)		(n=80)	(n=8)	
Gender				0.5260			0.4455
Male	82 (93.2)	73 (83.0)	9 (10.2)		75 (85.2)	7 (8.0)	
Female	6 (6.8)	5 (5.7)	1 (1.1)		5 (5.7)	1 (1.1)	
Ethnicity				0.7117			0.2572
Chinese	57 (64.8)	50 (56.8)	7 (8.0)		50 (56.8)	7 (8.0)	
Asian	29 (33.0)	27 (30.7)	2 (2.3)		28 (31.8)	1 (1.1)	
Undetermined	2 (2.3)	1 (1.1)	1 (1.1)		1 (1.1)	1 (1.1)	
Risk of Infection				N/A			N/A
IDU	18 (20.5)	17 (19.3)	1 (1.1)		17 (19.3)	1 (1.1)	
Homosexual	7 (8.0)	7 (8.0)	--		7 (8.0)	--	
Heterosexual	2 (2.3)	2 (2.3)	--		2 (2.3)	--	
Undetermined	61 (69.3)	52 (59.0)	9 (10.2)		54 (61.4)	7 (8.0)	
HIV-1 subtypes				0.0662			0.1006
B	18 (20.5)	13 (14.8)	5 (5.7)		14 (15.9)	4 (4.5)	
CRF01_AE	42 (47.7)	39 (44.3)	3 (3.4)		40 (45.5)	2 (2.3)	
C or CRF07_BC or CRF08_BC	14 (15.9)	13 (14.8)	1 (1.1)		13 (14.8)	1 (1.1)	
PCR -ve	14 (15.9)	13 (14.8)	1 (1.1)		13 (14.8)	1 (1.1)	
HCV genotypes				0.1267			N/A
1 / 1a / 1b	27 (30.7)	26 (29.5)	1 (1.1)		27 (30.7)	--	
3a / 3b	23 (26.1)	18 (20.5)	5 (5.7)		18 (20.5)	3 (3.4)	
6a / 6d / 6e	21 (23.9)	19 (21.6)	2 (2.3)		20 (22.7)	1 (1.1)	
PCR -ve	17 (19.3)	15 (17.0)	2 (2.3)		15 (17.0)	2 (1.1)	

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.

1A: rs12979860 homozygous wild-type (C/C) and mutant (T/T) detection



1B: rs8099917 homozygous wild-type (T/T) and mutant (G/G) detection

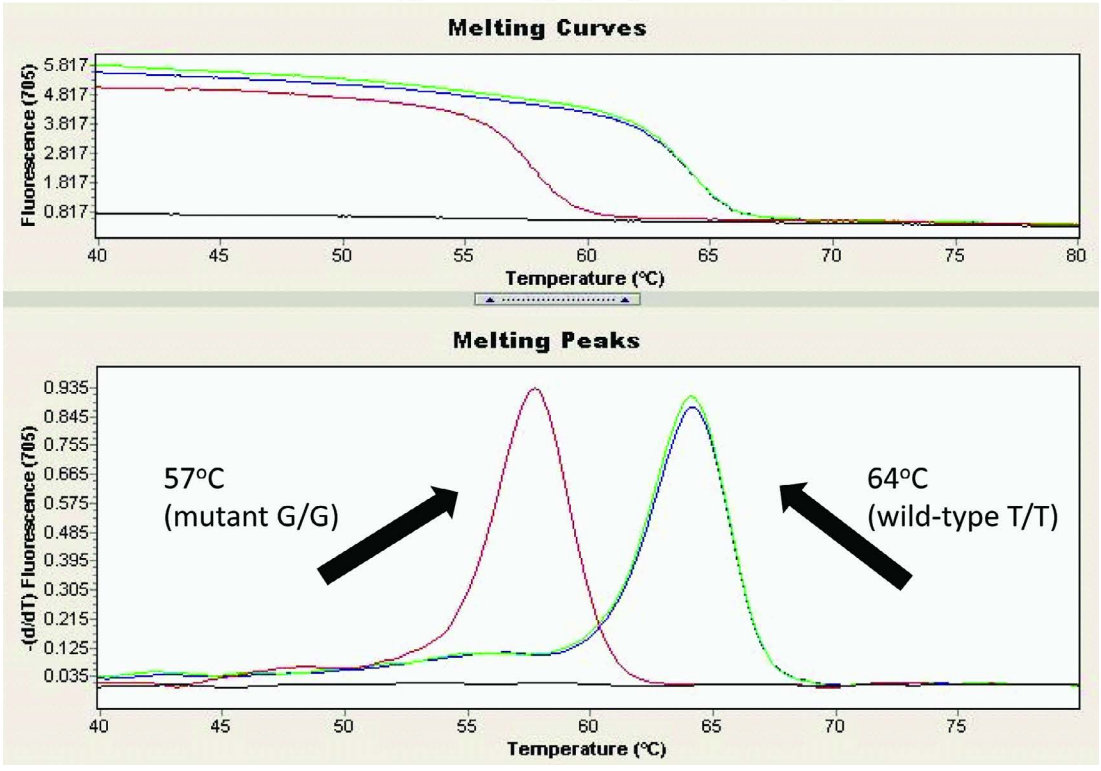


Figure 1: Melting curves and melting peaks analysis of rs12979860 (Figure 1A) and rs8099917 (Figure 1B) single nucleotide polymorphisms detection by in-house HybProbe assay. Heterozygous mutants could be interpreted when both melting peaks or wild-type and mutant were seen.

concordantly detected two C/T and one T/T mutants of rs12979860, as well as one G/T and one G/G mutants of rs8099917.

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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