High-resolution melting assay for genotyping of IFNL4-associated dinucleotide variant rs368234815

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Abstract

Interferon-λ4-related dinucleotide variant rs368234815 TT/-G is strongly linked with rs12979860 polymorphism, the most important genetic marker connected to treatment-induced hepatitis C virus clearance. Due to additional baseline information that rs368234815 polymorphism could provide for the management of chronic hepatitis C, we developed and validated a high-resolution melting genotyping assay using 193 patients with chronic hepatitis C.

Keywords: hepatitis C virus, high-resolution melting, *IFNL4*, *IL28B*, RT-PCR
Original Submission: 22 October 2013; **Revised Submission:** 18 February 2014; **Accepted:** 26 March 2014
Editor: G. Antonelli
Article published online: 11 April 2014
Clin Microbiol Infect 2014; **20**: O936–O938
10.1111/1469-0691.12637

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Genome-wide association studies showed that the single nucleotide polymorphism rs12979860 near to interleukin-28B gene (*IL28B*) was the most important genetic marker of spontaneous and interferon-induced clearance of hepatitis C virus (HCV) [1–4]. However, whether rs12979860 exerts biological effects or whether it is in linkage disequilibrium with other functional polymorphisms was unknown. Recently, two publications have described the identification of a novel dinucleotide variant rs368234815 TT/-G (previously designated as ss469415590) that is in strong linkage disequilibrium with the rs12979860 polymorphism and probably has a functional mechanism. In the first study Prokunina-Olsson *et al.* [5] showed that the rs368234815[-G] allele, which marks the unfavourable rs12979860[T], by frameshift mutation creates the new interferon-λ4 (*IFNL4*) gene encoding the IFN-λ4 protein, which could be a direct indicator of HCV IFN-based treatment failure by weak induction of the IFN-stimulated genes. In the second study [6] the rs368234815 TT/-G polymorphism was also found to promote the methylation of a cytosine residue and affect both IL28B and IFN-γ-inducible protein 10 mRNA expression in peripheral blood mononuclear cells stimulated with the dsRNA analogue poly(I:C). Both studies shared the conclusion that rs368234815 TT/-G polymorphism is a better marker for HCV clearance than rs12979860. More recent publications [7–10] confirm the association of *IFNL4* rs368234815 with both IFN-induced and spontaneous clearance of HCV infection, so we developed a real-time genotyping assay with detection based on post-PCR high-resolution melting (HRM) analysis by using a well-characterized cohort of 193 chronic hepatitis C patients treated with pegylated IFN-α and ribavirin (pegIFN-α/RBV) for 48 weeks [11,12].

High-resolution melting analysis is a fast and powerful technique able to distinguish wild-type and mutant variants in the presence of DNA binding dye that does not interact with ssDNA, but fluoresces strongly in the presence of dsDNA. Notably, the HRM method in terms of cost per sample is lower than other genotyping technologies, such as sequencing and hydrolysis single nucleotide polymorphism analysis (TaqMan).

To design the HRM assay, we considered that the amplicon around the mutation site should be possibly not longer than 100 bp and that the 1 bp difference must result in visible melting temperature differences, as far as possible >0.1°C. Yet, we also took into account the presence of two variants near to rs368234815 target site (rs73555604 and rs4803221) [5] that could affect the results of HRM genotyping assay in unexpected ways. Hence, we designed the primers both to limit the amplicon length to a short 63-bp region and to include the two variants surrounding the rs368234815 polymorphism site as degenerate positions (Table 1).

The HRM assay was evaluated using nucleic acid samples extracted from peripheral blood mononuclear cells of the 193 patients by the phenol–chloroform method. Patients gave written informed consent for participation in this study, which was approved by the local Institutional Review Board of the Department of Internal Medicine, operated according to the principles outlined in the Declaration of Helsinki. All extracted DNA samples were analysed for purity and yield via absorbance spectroscopy (A260/A280) using the NanoDrop ND

TABLE 1. Primer sequences for high-resolution melting assay, presequencing PCR and cycle sequencing

Primer	Nucleotide sequence (5′–3′)	Amplicon length (bp)
rs368234815HRM-F	CTGTGGGTCCTGT**R**CACGGT	63
rs368234815HRM-R	CGGTAGTG**S**GAGAGCAGGCA	
rs368234815Seq-F[a]	TCACGCTCCGAGCATTGCCT	903
rs368234815Seq-R	GGCCGCTGAGCACTGCCT	

Degenerate nucleotides for rs73555604 and rs4803221 variants are in bold: **R** = G or A; **S** = C or G.
[a]Cycle sequencing primer.

1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Real-time PCR and HRM were carried out using a Light-Cycler Nano System (Roche, Mannheim, Germany) on 20 μL of reaction mixture containing 1× LightCycler® high-resolution Master kit with LightCycler® 480 Resolight Dye (Roche, Mannheim, Germany), 0.2 μM each primer (rs368234815HRM-F and rs368234815HRM-R), 2.5 mM $MgCl_2$ and 1 μL of 10 ng/μL of the extracted DNA as input. Importantly, to achieve the best results, the amount of input DNA should be adjusted to be similar for all samples within any one run. The thermal cycling conditions include an initial hold at 95°C for 10 min, followed by 45 cycles of 95°C for 10 s (denaturation), 60°C for 15 s (annealing), and extension step of 72°C for 20 s. Acquisition of the fluorescence signal was performed during each extension step. Amplification was followed by HRM analysis of the real-time PCR products, consisting of a denaturation step at 95°C for 60 s, a cooling step at 40°C for 60 s, a short hold at 60°C for 1 s and a continuous acquisition step from 60°C to 95°C at a ramp rate of 0.3°C/s. By using specific setting ranges for pre-melt and post-melt temperatures of 81.1–84.1°C and 92.5–94.4°C, respectively, it was possible to distinguish clearly the rs368234815 TT/TT, TT/-G and -G/-G genotypes in the normalized difference plot (Fig. 1).

The genotyping success rate of rs368234815 by HRM assay was 100% and no deviation from Hardy–Weinberg equilibrium ($p > 0.05$) was observed. Sanger sequencing control performed with Seq-primers (Table 1) on 100 randomly picked samples, obtained a 100% concordance with genotypes from HRM. The genotypic frequencies of rs368234815 variant (37% for TT/TT, 49.5% for TT/-G and 13.5% for -G/-G) matched that of rs12979860 polymorphism (37% for CC, 50% for CT and 13% for TT) achieved previously by TaqMan assay [12], confirming their high linkage disequilibrium (Table 2). As the only patient with mismatched genotype was a non-responder to combined treatment, the predictive value of the *IFNL4* rs368234815 and rs12979860 variants is not different in our cohort.

Treatment of chronic hepatitis C infection is rapidly evolving towards IFN-free, direct-acting antiviral (DAA)

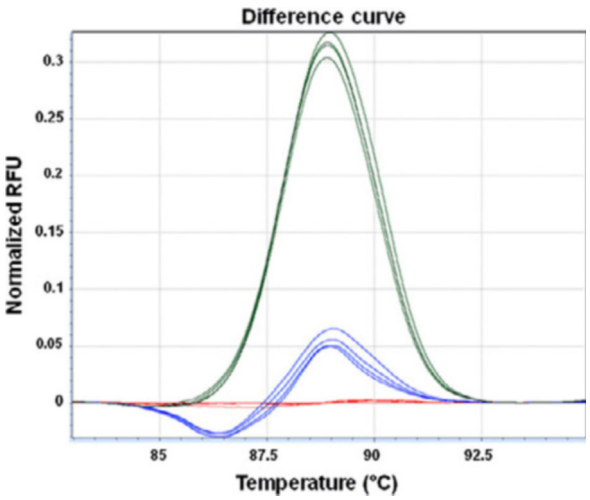

FIG. 1. Representative difference graph for DNA samples from hepatitis C virus (HCV) infected patients carrying different rs368234815 genotypes (TT/TT: red lines, TT/-G: blue lines and -G/-G: green lines), was achieved using the LightCycler® NanoSW1.1 software (Roche, Mannheim, Germany) by subtracting the normalized fluorescence data of the TT/TT genotype, selected as a baseline, from that of each of the other samples in the high-resolution melting analysis. The position of each sample relative to the baseline is plotted against the temperature to generate a difference plot that helps to cluster samples manually into groups that have similar melting curves (e.g., those with the same genotype).

TABLE 2. Genotype distribution and linkage disequilibrium of rs368234815 and rs12979860 variants

	TaqMan assay for rs12979860			
	CC	CT	TT	Total
HRM assay for rs368234815				
TT/TT	72	0	0	72
TT/−G	0	95	0	95
−G/−G	0	1	25	26
Total	72	96	25	LD[a] (r^2 = 0.98)

[a]The r^2 coefficient of LD was calculated as described elsewhere [17].

regimens that specifically target the HCV protease (NS3/4A) (e.g. Telaprevir and Boceprevir), RNA polymerase (NS5B) (e.g. Sofosbuvir), or non-structural protein NS5A (e.g. Daclatasvir). Although phase 2 and 3 studies of all oral IFN-free regimens have repeatedly shown optimal efficacy and tolerability rates [13], access to these therapies is unlikely to be universal in many countries because of cost issues. The ability to predict treatment efficacy and rate of HCV RNA decline during Telaprevir- and Sofosbuvir-based therapies using the *IFNL4* genotype could therefore be clinically useful [14,15], as prediction of efficacy of IFN-based regimens might still have

a place for treatment allocation. Nevertheless, at the current time, the rs368234815 variant is not considered a key diagnostic test in the treatment decision algorithm [16].

In summary, we developed a sensitive, cost-effective HRM genotyping assay for the novel *IFNL4* rs368234815 polymorphism that can be adopted only in virological laboratories with HRM capability to provide additional baseline information for the management of patients with chronic hepatitis C.

Transparency Declaration

The authors declare no conflicts of interest.

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