protein A1), also showed a low correlation coefficient of 0.330, suggesting that its usefulness was limited in HBV positive oriental patients. Although FIB-4 demonstrated the best coefficient of 0.412 among the fibrosis scores, significant overlaps were found between neighboring stages and obtained scores were not coordinated for real histological classification.

In conclusion, the FSB was a useful and reliable biomarker for prediction of liver fibrosis in patients with chronic HBV infection. The FSB is expected to be introduced and utilized in varied kinds of studies and trials. Its accuracy and reproducibility require further validation using higher numbers of patients in several countries other than Japan.

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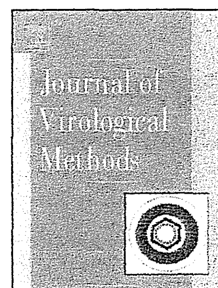

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Comparative quantitative analysis of hepatitis C mutations at amino acids 70 and 91 in the core region by the Q-Invader assay

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Abstract

Hepatitis C virus (HCV) is a major worldwide public health problem, and mutations at amino acids 70 and 91 in the genotype 1b core region predict the effectiveness of combination therapy with peginterferon and ribavirin. An assay based on the Q-Invader technology was developed to determine the relative ratios of the mutant to wild-type virus with high sensitivity. The assay detected a minor type plasmid that constituted only 1% of a mixture of plasmids containing wild-type and mutant sequences. The calculated ratios agreed with those of the template DNA. A total of 123 serum samples of HCV in Japan were examined with the Q-Invader assay. The Q-Invader assay detected all of the mutations that were detected by direct sequencing and even some mutants that direct sequencing could not. PCR with mutant specific primers confirmed those mutations found by the Q-Invader assay and not by direct sequencing. The Q-Invader assay, thus, is a useful tool for detecting mutations at positions 70 and 91 in the HCV-1b core region.

1. Introduction

Hepatitis C virus (HCV) affects approximately 170 million people and is a major worldwide public health problem. It is responsible for chronic liver disease and increases the risk for severe diseases, such as cirrhosis and hepatocellular carcinoma (HCC) (Seeff et al, 2002). Interferon (IFN) therapy in chronic hepatitis C infection reduces the risk of developing HCC and liver-related death by clearing the virus. Since 2004, combination therapy with peginterferon (PEG-IFN) and ribavirin (RBV) has been the standard treatment (Fried et al, 2002). Recently, a triple therapy of peg-interferon, ribavirin and a direct-acting antiviral (DAA), such as the protease inhibitor boceprevir or telaprevir, was approved and has been routinely used since 2011 (Kumada et al, 2012). However, for some patients, a sustained virological response does not last long. In Japan, the most common HCV genotype is genotype 1b (about 70%), but no virological response was found in 26.3% of patients infected with genotype 1b (Akuta et al, 2006).

In the present study, two key factors were related to the lack of a virological response in patients with the HCV genotype 1b: host polymorphisms at the neighboring IL28B gene (Ge et al, 2009; Tanaka et al, 2009; Thomas et al, 2009) and mutations in the HCV-1b core region (Akuta et al, 2005, 2006). The HCV-1b mutations (e.g., arginine to glutamine or histidine at position 70 and/or from leucine to methionine at position 91) were significantly more common in virological non-responders, and the rate of decline in the HCV load during combination therapy in patients with mutant variants was less than those of patients with wild-type virus (Akuta et al, 2005). With the triple therapy, the proportions of sustained virological response were predicted by the prevalence of mutations at positon 70 and the polymorphisms at the neighboring IL28B gene (Akuta et al, 2010). In addition, the prevalence of mutations at position 70 was increased by positive selection during combination therapy (Kurbanov et al, 2010). Therefore, for HCV therapy, monitoring the ratios of mutant variant to wild-type at position 70/91 may be as important as monitoring the HCV load. However, measuring the ratios of one point mutation with high sensitivity is difficult because only one quantitative method is available (Nakamoto et al, 2009).

This report describes a novel approach that uses the real-time PCR monitoring Invader reaction (Q-Invader assay) in a comparative quantitative assay for mutations at positions 70/91 in HCV-1b core region. The Invader technology has high specificity for detecting single-nucleotide differences in genomic DNA or PCR products (Lyamichev et al., 2000), and a highly sensitive quantification assay can be attained by adding

real-time PCR (Tadokoro et al, 2009, 2010). To demonstrate its sensitivity and effectiveness, the method was used to detect mutations in clinical samples, and the results were compared to those from sequencing (Akuta et al, 2005) and PCR with mutation-specific primers (Okamoto et al, 2007).

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2. Materials and methods

2.1. Source of patients

Serum samples were obtained from 123 patients infected with HCV-1b at the Toranomon Hospital (Kanagawa, Japan). The study was conducted in accord with the ethical principles of the Declaration of Helsinki and was approved by the Toranomon Hospital Ethical Committee. Written informed consent was obtained from each patient.

2.2. Design of Invader probes

The primary probe and Invader oligo to detect mutations (i.e., R70H/Q and L91M in the core region) were designed with the Invader technology creator (TWT, Madison, WI, USA) (Figure 1 and Table 1). Variations in neighboring regions were confirmed by analyzing 55 individual sequences of the HCV core region from a database at the National Center for Biotechnology Information (NCBI).

2.3. Extraction HCV RNA and cDNA synthesis

HCV RNA was extracted from 200 μl of serum and eluted in 10 μl RNAse/DNAse-free water with the PureLink Viral RNA/DNA Mini Kit (Life Technologies, Carlsbad, CA, USA). cDNA was synthesized with random primers in 20-μl reactions with a commercial kit (SuperScript III cDNA Synthesis Kit, Life Technologies).

2.4. The Q-Invader assay

Comparative quantitative analysis of mutant and wild-type virus was completed by the Q-Invader assay. Two fluorescence signals (carboxyfluorescein or FAM for wild-type; REDmond RED or RED for mutants) could be detected in a single reaction with a Universal General Purpose Reagent (TWT), including Cleavase and FRET mix with two common fluorescence probes. Template c-DNA was added to a 15-μl reaction mixture containing 500 nM primers for amplification HCV-1b core region, 300 nM of each primary probe, 700 nM Invader oligo, 2 U AmpliTaq gold (Life Technologies), Universal General Purpose Reagent (TWT) and FRET mix (Table 1). The reaction mixture was preheated in a 384-PCR plate (Roche, Basel, Switzerland) at 95°C for 20

min, and a two-step PCR reaction was carried out for 40 cycles (95°C for 15 sec, 65°C for 60 sec) in a LightCycler 480 (Roche) (Tadokoro et al, 2009). Fluorescence values of FAM (wavelength/bandwidth: excitation, 465 nm; emission, 510 nm) and RED (excitation, 533 nm; emission, 610 nm) were measured at end of the incubation/extension step at 65°C for each cycle and by standard real-time PCR. By analyzing the results, a crossing point (Cp) can be obtained by a fit point method (Luu-The et al., 2005) in the LightCycler 480 software.

2.5. Sequencing

The sequence at position 70/91 in clinical samples was determined as described (Akuta et al, 2005). The amplified products were sequenced by the dideoxy method with the BigDye Terminator v1.1 Cycle Sequencing Kit (Life Technologies) in a model 3130 fluorescent DNA sequencer (Life Technologies).

2.6. Manufacture of control plasmid DNA by cloning clinical HCV sequence

HCV RNA, wild-type and that with mutations at positions 70/91, was isolated from clinical samples and amplified by. PCR amplicons were cloned into the pCRII-TOPO vector (Life Technologies) and sequenced (Tadokoro et al, 2009).

2.7. Mutation analysis by wild-type/mutant-specific primers

The mutations of positions 70/91 in patient c-DNA were determined by PCR with wild-type/mutant specific primer (Okamoto et al, 2007). The PCR amplicon was confirmed by electrophoresis with a MultiNA microchip electrophoresis system (Shimadzu, Kyoto, Japan).

2.8. Detection sensitivity and assay variation for the Q-Invader assay

Detection sensitivity and assay variation for the Q-Invader assay were examined with single or mixed plasmids for template DNA. The detection limits of the Q-Invader assay in each measurement were determined with a 10^{-7} dilution of plasmid. Mixtures of plasmids were prepared in various ratios and determined in the Q-Invader assay for comparative quantitative analysis. A fivefold measurement was performed three times each to examine assay variation.

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3. Results

3.1. Detection threshold and assay variation

Measurements of the mutant and wild-type virus at position 70 were both effective between 10^1 and 10^7 copies. The detection thresholds of both measurements were 10^1 copies of HCV c-DNA. Measurements at position 91 (mutant and wild-type) were effective between 10^2–10^7 copies, and the detection threshold was 10^2 copies (Fig. 2 and Fig. 3).

3.2. Comparative quantitative analysis for calculating relative ratios

Comparative quantitative analysis was used to determine the relative ratios in competitive infections by mutant variant and wild-type virus. Copy numbers of mutant and wild-type virus were calculated separately by fit point analysis with each standard plasmid. To determine the relative ratios, the number of mutants was divided by those of total (mutant + wild-type). When the mutant and wild-type plasmids were mixed in various ratios for template DNA, the minor plasmid (mutant or wild-type) in template DNA could be detected down to 1:100 (Fig. 4), and the calculated relative ratios agreed with those of template DNA (Fig. 5).

3.3. Determination of relative ratios in clinical samples

Sequences from the HCV core region of 123 serum samples were confirmed by direct sequencing. The Q-Invader assay was used to determine the relative ratios of mutants at positions 70/91. The relative ratios identified three categories to compare by direct sequencing: less than 1% (wild-type), 1–99% (mixed) and more than 99% (mutant). Mixed types were found in 37 samples (30.1%) at position 70, and only 43.2% (16 of 37) of the cases were detected by the Q-Invader assay. At position 91, 22 samples (17.9%) were found to be of the mixed type, and only 59.1% (13 of 22) of the cases were detected by Q-Invader assay.

3.4. Confirming mixed type by PCR with specific primer

To confirm the mixed type decided by the Q-Invader assay, but not by direct sequencing, PCR with wild-type/mutant specific primer was performed for each four

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samples. At position 70, a minor mutant variant was detected in samples 99 and 118 (4% and 5%). In samples 80 and 18, mutant variant held most (98% and 99%). By PCR with specific primer, both wild-type and mutant were detected in all samples. A similar result was obtained at position 91 (Table 3).

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4. Discussion

Accurate, rapid determination of the relative ratios of mutations at positions 70/91 in the HCV-1b core region is important to insure the effectiveness of the combination therapy with peginterferon and ribavirin. A highly sensitive method with comparative quantitative analysis was developed in this study. The Q-Invader assay was examined for detection sensitivity and accuracy and compared with direct sequencing and PCR with type-specific primers.

Multiple reaction systems with two florescence probes were examined for detection of wild-type/mutant sequences at two positions (70 and 91) in the HCV-1b core region. The detection sensitivities at position 70, both wild-type and mutant, were 10 copies of template DNA, and those at position 91 were 100 copies (Fig. 3). In examinations of mixtures of plasmids with wild-type and mutant sequences in various ratios, the Q-Invader assay could be determined down to 1% of the minor type (Fig. 4), and the calculated relative ratios agreed with those determined by use of template DNA. The relative ratios were not influenced by total number of DNA templates (Fig. 5).

To validate the Q-Invader assay, 123 clinical samples from patients were analyzed for mutations at positions 70/91 in HCV-1b core region by direct sequencing and the Q-Invader assay. All of mutations found by direct sequencing were also detected by the Q-Invader assay. Moreover, The Q-Invader assay could detect wild-type/mutant that slightly included in sample. At position 70, both wild-type and mutant were detected in 37 samples by the Q-Invader assay and 56.8% (21 of 37) of the cases could detect only either one by direct sequencing. To confirm the existence of the minor type detected by the Q-Invader assay, the PCR with specific primer was performed (Table 3).

The relevance of amino acid mutations at positions 70/91 in HCV-1b core region to the effectiveness of combination or triple therapy was reported in many studies (Akuta et al, 2005, 2010; Nakamoto et al, 2010). In addition, amino acid mutations at the positions 70/91 were resistant to interferon *in vitro* (Funaoka Y et al, 2011). The expression levels of IL-6, which upregulates SOCS3, in cells transfected with the core mutant were significantly higher than with wild type. These mechanisms may explain the clinical resistance of amino acid mutations at positions 70/91 for interferon therapy. Furthermore, the amino acid mutations at position 70 in the HCV-1b core region are significant as an independent predictor of HCC in virological non-responders (Seko et al, 2012).

In studies of interferon therapy and HCC development, the importance of the amino acid mutations at positions 70/91 is increasing. In addition, the relative ratios of mutants

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varied in individual patients (Okamoto et al, 2007) and changed between therapies (Kozuka et al, in press; Kurbanov et al, 2010). Therefore, monitoring the relative ratios of mutant variants should contribute to new knowledge and efficacy prediction for HCV therapy. A high-quality quantitative system is required to push forward a study. The Q-Invader assay, which is more high sensitive method than existing method, would provide detailed dynamic change of relative ratios.

In summary, the Q-Invader assay had a high sensitivity for calculating the relative ratios of mutant variants at positions 70 and 91 in HCV-1b core region. The mutant variants were detected down to 1% of the total, and mixed template DNAs in various ratios were quantified accurately by the Q-Invader assay. The Q-Invader assay will be useful for patients with HCV in clinical setting.

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Figure Legends

Fig. 1. Cleavage point for the Q-Invader assay at position 70 in the HCV-1b core region. White boxes represent cleavage points in PCR amplicon. Reference sequence: HCV-J.

Fig. 2. Amplification plots generated by the Q-Invader assay. Fluorescence intensity was plotted against the number of cycles for a 10^{-7} dilution of plasmid. (W: wild-type plasmid DNA, M: mutant plasmid DNA)

Fig. 3. Standard curves generated by the Q-Invader assay. The Cp was calculated by the fit point method with a 10^{-7} dilution of plasmid.

Fig. 4. Detection threshold in the Q-Invader assay with plasmid DNA at ratios of 100:1 to 1:100. At position 70, the minimum number of minor types was 10 copies. Position 91 was 100 copies.

Fig. 5. Assay variation in the Q-Invader assay with 0–100% mixed plasmid DNA. Plasmid DNA was mixed to 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% with the mutation. At position 70, total numbers of plasmid DNA copies were 10000 (A) and 100 (B). Position 91 were also 10000 (C) and 1000 (D) copies.

Table 1

Primer and Invader probe with the Q-Invader assay

Target		Sequence(5'-3')
core 70	F-primer	CCTCGTGGAAGGCGACAACCTAT
	R-primer	GGCCADGGRTACCCRGGCTG
	p1 probe	CGCGCCGAGG**C**GRMRAKCYTTGG
	p2 probe	ACGGACGCGGAG**T**GRMRAKCYTTGG
	io probe	GCCCAGGHYCTRCCCTCGKBNA
core 91	F-primer	CCTGGGCTCAGCCYGGGTA
	R-primer	CGGGGTGACAGGAGCCATC
	p1 probe	CGCGCCGAGG**Y**TRGGRTGGRCAG
	p2 probe	ACGGACGCGGAG**A**TRGGRTGGRCAGGAT
	io probe	TTGGCCCCTCTAYGGCAAYKAGGGYT

F-primer=forward primer; R-primer=reverse primer; p1 probe=primary probe (FAM); p2 probe=primary probe (RED); io probe=Invader oligo

Underlined sequence represents the 5' flap of probe

Amino-blocked 3' end of all primary probes

Boldfaced sequences denote the cleavage site of primary probes.

Table 2

Comparison of the number of mutant by the Q-Invader assay with those of sequencing

Core 70

		Sequencing: Wild	mixed	Mutant
Q-Invader (Mutant%)	Less than 1%	43		
	1-99%	12	21	4
	more than 99%			43

Core 91

		Sequencing: Wild	mixed	Mutant
Q-Invader (Mutant%)	Less than 1%	55		
	1-99%	9	9	4
	more than 99%			46

Table 3

Confirmation for mixed type by PCR with specific primers

Core 70

	Q-Invader		PCR with specific primers	
	Mutant(%)	Sequencing	Wild	Mutant
Sample 099	4%	Wild	+	+
Sample 118	5%	Wild	+	+
Sample 080	98%	Mutant	+	+
Sample 018	99%	Mutant	+	+

Core 91

	Q-Invader		PCR with specific primers	
	Mutant(%)	Sequencing	Wild	Mutant
Sample 088	2%	Wild	+	+
Sample 067	2%	Wild	+	+
Sample 090	98%	Mutant	+	+
Sample 071	98%	Mutant	+	+

Figure(s)

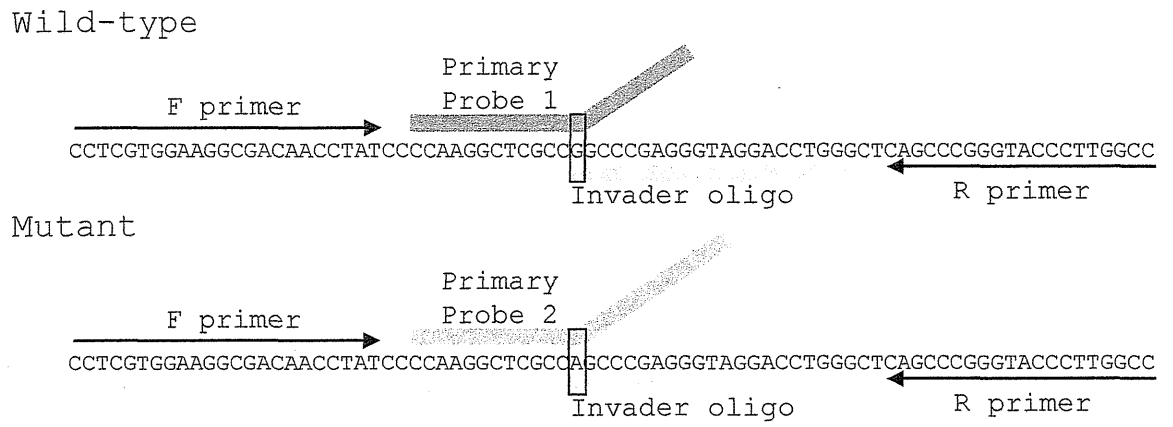
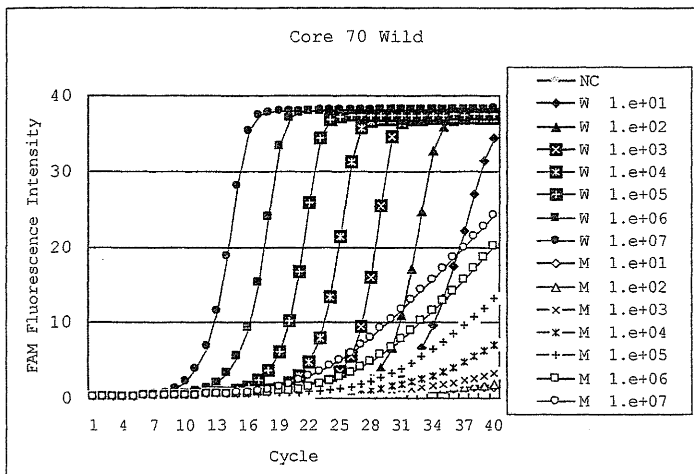
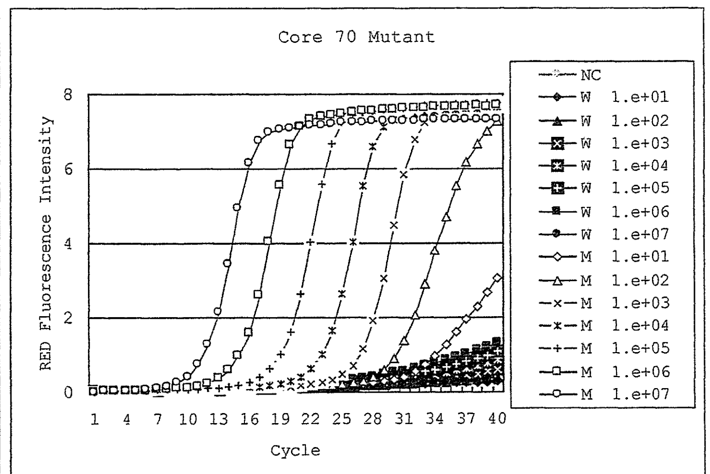
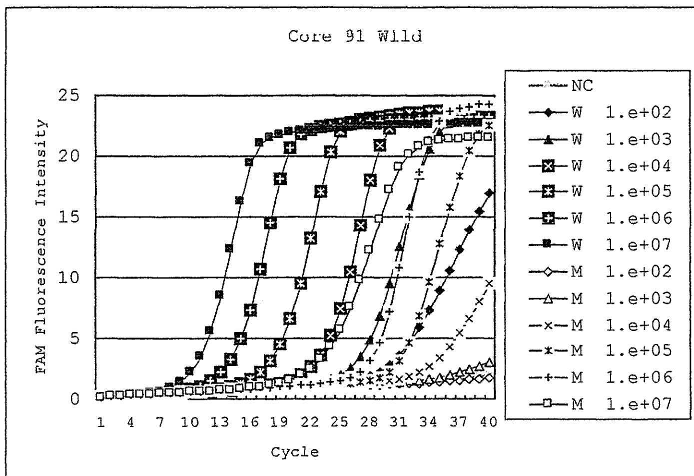


Figure 1.







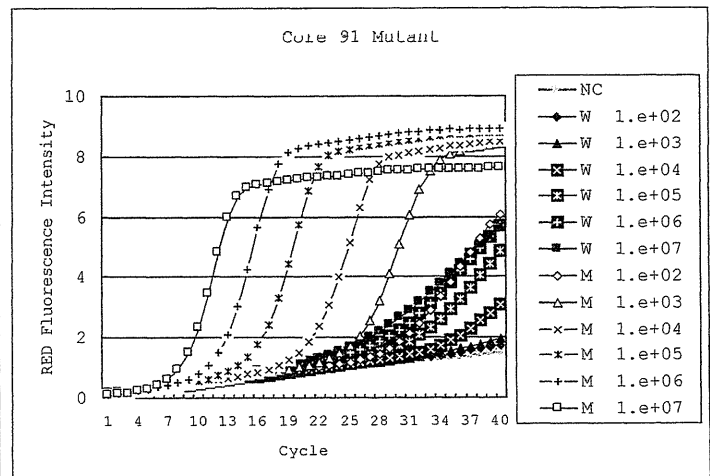


Figure 2.