Screening for genetic heterogeneity in the interferon sensitivity determining region of the HCV genome by polymerase chain reaction with melting curve analysis.

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Running head: simple MCA for screening of mutations in the ISDR

Abstract

Background and Aim: Although mutations in the interferon (IFN) sensitivity determining region (ISDR) of HCV have been reported to be useful as a predictive viral factor for IFN therapy in patients infected with HCV-1b, such laboratory research has not been favorably translated into the clinic. To promote such translation, we attempted the establishment of a rapid and simple polymerase chain reaction (PCR) combined with melting curve analysis (MCA) to screen for mutations in the ISDR and for the monitoring of HCV-quasi-species. Methods: A PCR-MCA protocol was established using in-house primers and hybridization probes designed according to the results of direct sequencing of 34 HCV-1b samples. Then, the performance of PCR-MCA was verified by comparing with mutation profiles obtained by direct sequencing and sequencing after cloning. Results: The MCA assay revealed that melting temperature (Tm) was inversely correlated with the number of nucleotide (nt) and amino acid (aa) substitutions in the ISDR deduced on the basis of the results of direct sequencing. A boundary Tm of 58.0℃ allowed us to discriminate HCV genomes into two groups; one with Tm>58.0℃ had no- or low number of nt substitution, whiles the other genomes with Tm<58.0℃ had high number of nt substitution, corresponding to wild-type in the former and mutant-type in the latter in respect of a clinical setting for IFN therapy. Moreover, this MCA assay provided precise discrimination of Tm between clones, reflecting the degree of the genetic complexity of HCV genomes. Conclusion: This study indicates that the MCA assay is useful to rapidly and simply screen the mutational status of the ISDR region of HCV as well as in using ISDR as one of targets for discriminating the genetic complexity of HCV genomes. The MCA assay also could be applicable as a convenient and useful screen of the genetic heterogeneity of clones relating to HCV quasi-species.

Key words: ISDR, HCV, IFN, MCA, quasi-species

List of Abbreviations

ISDR; Interferon sensitivity determining region

PCR; polymerase chain reaction

HCV; hepatitis C virus

MCA; melting curve analysis

IFN ; interferon

SSCP; single strand conformation polymorphism

Tm;melting temperature

Introduction

Hepatitis C virus (HCV) is a RNA virus belonging to the Flaviviridae. It has a single-strand plus genome of about 9.6 kb with a single open reading frame encoding four structural (C, E1, E2, and p7) and six non-structural (NS2, NS3, NS4A, NS4B, NS5A ,and NS5B) proteins[1)]. HCV is persistently present in plasma as a mixture of heterogeneous HCV RNA genomes, quasi-species, and causes chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) in humans. Patients with HCC have been reported to be prevalent in the Nagasaki district and a majority of them are sero-positive for the virus[2)]. Fortunately, recent advances in the treatment of chronic hepatitis C are promising, namely interferon-ribavirin combination therapy as the mainstay treatment for chronic hepatitis C. However, such combination therapy is not always effective in all patients with HCV-related disorders, and can also cause adverse events associated with the drugs[3, 4)]. Recently, the HCV core antigen is generally used to quantitatively monitor HCV viremia, but not appropriate for the monitoring of

the complexity of HCV genome relating to HCV quasi-species. For these reasons, a bio-marker to predict the efficacy of interferon (IFN) therapy is required. At present, the presence of HCV genotype-2 or -3 and a low viral load prior to treatment have been proposed and applied in a practical setting as predictive markers for the effectiveness of IFN therapy[5]. Although HCV genotype 1b is resistant to IFN therapy, Enomoto et al[6] reported that mutations in the interferon sensitivity determining region (ISDR) within the NS5A gene are useful as predictive markers of the response to IFN therapy for chronic hepatitis patients with a Japan-specific subtype (J-type) of the HCV genotype 1b. The ISDR corresponding to codons 2209-2248 of the HCV genome (NS5A2209-2248) can interact with the double-strand RNA protein kinase (PKR). This interaction has been shown to be able to block the IFN signaling pathway, leading to HCV resistance to IFN therapy[7, 8]. However, the precise mechanism of IFN resistance remains unresolved because the regions other than ISDR, such as E2, NS3/4, and NS5A, have also been reported to be involved in resistance to IFN therapy in Japanese patients[9, 10].

Mutations in the ISDR that are expected to be useful as predictive markers have not yet been introduced into the clinical setting. One of the reasons is due to the methodology used for routine laboratory tests is inapplicable because of the labor-, time-, and cost-consumed.

In the present study, to evaluate the implications of nucleotide substitutions within the ISDR region in the resistance to IFN therapy and in quasi-species, we examined genetic heterogeneity in the ISDR of HCV isolates from clinical samples. Then, we attempted the development of a rapid and easy methodology for screening of nucleotide mutations using polymerase chain reaction (PCR) with melting curve analysis (MCA).

Materials and Methods

Study samples

HCV samples were collected from plasma specimens from patients admitted to our hospital. Clinical information was referred from the hospital information system, as summarized in Table 1. HCV genotyping was determined with multi-plex PCR, as described by Ohno et al.[11]. Quantification of serum HCV RNA and HCV antigens were performed according to the manufacturer's instructions using a commercial kit (Ortho HCV Antigen IRMA Test; Ortho Clinical Diagnostics, Raritan, NJ, USA). HCV isolates with genotype type-1b classified according to the typing system by Ohno et al.[11] were enrolled in this study. Clinical virological assay for the qualification and quantification was done by using commercial assay kits (AMPLICORE HCV Monitor test, version 2.0 and AMPLICORE HCV, version 2.0, Roche Diagnostics, Basel, Switzerland).

HCV RNA extraction, amplification by nested PCR, and sequencing

HCV RNA was extracted from serum samples using a QIAamp Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. cDNA was generated using SuperScript III Reverse Transcriptase (Invitrogen Corp., Calsbad, CA, USA) and antisense primer or random hexamer oligonucleotides. A fragment of 251 bp including the ISDR region was amplified by nested-PCR using primers, as described previously[12]. The amplicons were subjected to single strand conformation polymorphism (SSCP) and direct sequencing analysis in order to identify mutations in the ISDR. PCR-SSCP was performed according to the standard method[13, 14]. Direct sequencing was done with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) using an Automated DNA Sequence Analyzer(Model 310, Applied Biosystems). The nucleotide (nt) and deduced amino acid (aa) sequences of the ISDR region were aligned and compared with that of the HCV-J prototype[15]. From several samples, PCR products were cloned using a

DynaExpress TA PCR Cloning Kit with Jet Competent Cells (BioDynamics Laboratory Inc., Tokyo, Japan) and then each clone was sequenced and subjected to melting curve analysis (MCA) assay.

PCR-MCA

To screen for mutations in the PCR amplified products in the ISDR, melting analysis was applied using a Lightcycler instrument equipped with the DNA MCA analysis program (LIghtCycler Software Version 3.0)[16]. Briefly, reactions were performed in a 20 μl volume with 5 mM $MgCl_2$, 2 μl 1/10000 diluted PCR product, 0.5 μM PCR primers, 0.4 μM anchor and sensor hybridization probes (Table 2), and 2 μl of 10×LightCycler FastStart DNA Master HybProbe (Roche Diagnostics). After 35 amplification cycles, the PCR products were denatured at 95℃ for 20 seconds, annealed at 40℃ for 20 seconds, and then raised from 40 to 80℃ at a transmission rate of 0.2℃ during continuous fluorescence monitoring at 640 nm. The fluorescence intensity was analyzed using the manufacturer's program, generating melting temperature (Tm) and melting curve values.

The results by the MCA assay were validated by comparing with that of direct sequencing.

Results

1. Mutation in the ISDR and relevancy for probe setting

To evaluate the heterogeneity of mutations in the ISDR region (nts 6954 to 7073), a fragment of the region was amplified and sequenced directly for 34 HCV samples. Compared with a reference sequence of the HCV-1b prototype (Gene Bank accession D90208), the sites of nt substitutions were mainly distributed in two regions at or near nt 6974 and nt 7070, as shown in Figure 1. Although the site of silent mutations (open column) was widely distributed, the site of missense mutations (solid column) was concentrated into the region of nts

6960 to 6990 corresponding to codons 2211 to 2221. The number of aa substitutions corresponding to missense mutations was from 0 to 6; with no substitution (wild-type) in 15 cases, one in 9 cases, and 2 or all cases had at least one nts substitution with concentration of missense mutations within the region of nts 6954 to 6990, the probe site was targeted in the region, as shown in Table 2.more in 10 cases. Since The G: C content of the anchor probe was approximately 53.9%, and sensor probe was 46.7%. Using this probe, our PCR-MCA method gave reasonably sharp melting curves in all 34 cases with total run times of about 3 hours, as demonstrated below.

2. Performance of PCR with MCA discriminating heterogeneity in the ISDR

Since the Tm value is essentially regulated by heteroduplex formation depending upon various genetic and epigenetic alterations, we verified whether PCR with MCA can discriminate and screen the mutation status in the ISDR. As shown in Figure 2(A), Tm values of amplicons derived from blood samples varied from about 45 to 65℃, and were inversely correlated with the number of nt. Substitutions ($r=-0.689$, $P<0.01$) and the deduced aa substitution number ($r=-0.617$, $p<0.01$). In particular, Figure 2(B) reveals that the boundary of the Tm of 58.0℃ makes it possible to distinguish two major groups; a high Tm group of >58.0℃ and a low Tm group of <58.0℃. Samples distributed in the high Tm area had mainly no or rarely one aa substitution, whereas samples distributed in the low Tm area had mainly 2 or more aa substitutions. However, although only samples having one aa substitution were distributed to both the high and low Tm areas, the total number of nt substitutions was different between them; about two times higher in samples distributed in the low Tm area rather than the high Tm area.

Then, as shown in Table 3, viral and clinical parameters between the high and low Tm groups were compared. The high Tm group showed a tendency of low nt and aa mutations, high viral load, and non-response to IFN. Figure 3 shows

representative cases who received IFN therapy, showing that patients 15, 19 and 33 (upper panel) were non-responders with Tm = 59.3℃, 62.5℃ and 64.8℃, while patients 34 and 37 (lower panel) were responders with Tm = 56.1℃ and 45.7℃. A patient infected by HCV with Tm 62.1℃ was an exceptional responder. All of these suggest that the high and low Tm values are equivalent to wild-type and mutant-type ISDR from the viewpoint of the prediction of IFN efficacy.

3. HCV quasi-species and MCA

HCV is present in plasma as a mixture of heterogeneous RNA genomes relating to HCV quasi-species. First of all, we examined the genetic complexity of ISDR of HCV genomes by SSCP analysis. PCR-SSCP analysis revealed 2 to 7 bands (mean 5 bands, as shown in Figure 4), indicating that there were variant clones with differences in the degree of complexity even at the pre-treatment stage. Then, from 4 cases in lanes 3 (patient No. 17), 12 (No. 28), 20 (No. 37), and 1 (No. 15), a total of 86 clones were established and subjected to MCA assay. As shown in Figures 5 and 6 (B), MCA gave instructive Tm distributions forming several clusters with minor separated clones. The differences in Tm values among the clones were correlated to the nt substitution number, indicating that HCV genomes are heterogeneous in ISDR case by case. These findings on Tm for amplicons and clones and their cluster patterns are shown in Figure 5. For example, in patient No. 17, although the Tm of the amplicon was 57.2℃, the Tms of clones were variable with a range of 50.7℃ to 66.2℃ distributing in 2 major clusters around 50.8℃ and 57.8℃, one minor cluster, and scattered clones. The band number was 3. In patient No 15, changes in the composition of clones defined by Tm were monitored during IFN therapy, as shown in Figure 6. The Tm of 3 HCV samples collected at the 3 clinical points of pre-treatment, discontinuation, and re-treatment was almost the same, at 61.2℃, 60.7℃, and 61.0℃, respectively, whereas dot plot distribution profiles of Tm after cloning of each sample were subtly different with range of 51.0-63.4℃,

55.3- 60.7℃, and 51.6-62.3℃, respectively. Notably, among these clones with different Tm, clones with values less than 55.0℃ disappeared after treatment with IFN, and they appeared again at the time of the re-treatment. The major clones with high Tm remained unchanged.

Discussion

In this study, we developed a simple and rapid PCR-MCA method for screening the genetic heterogeneity of the ISDR of HCV genomes, indicating that this MCA assay is applicable for clinical use as a predictive marker of IFN efficacy or monitoring of the emergence of new clones. This is meaningful, because about 2% of the human population worldwide are infected with and suffer from HCV-related disorders. At present, IFN therapy makes it possible to relieve symptoms caused by HCV, such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma[17, 18]. However, the current combination therapy of IFN and ribavirin is not universally effective in patients with chronic hepatitis C, showing the response rates of 90% to 40%[19]. The difference in responsiveness is thought to be in part explained by viral load and the genetic heterogeneity of the HCV genome[5, 6, 20]. In particular, genotype 1 prevalent in Japan is known to be resistant to IFN. Enomoto et al [6] describe a correlation between the number of mutations within a 40 aa sequence of the ISDR region and the response to IFN therapy in genotype-1b infected patients. Thus, although the mechanism of IFN resistance is now being elucidated, the translation from research laboratory to clinical application is poor. The practical application of findings of ISDR mutations and IFN responsiveness is no exception. The reason why practical applications lag behind current research is due in part to technical and economic problems in translating clinical laboratory tests, with regard to time, labor and cost. Although there are many methodologies for the detection of mutations, such as sequencing, SSCP, DHPLC, micro-array technology, and so on, we

examined melting curve analysis combined with PCR amplification, because it is a simple and rapid method for screening of genetic alterations. MCA is based on the property that the melting temperature of duplex DNA is dependent upon the length, sequence, G:C content, and Watson-Click base pairing[21,22)].

As the Tm value precisely reflects genetic alterations in the ISDR, a sequence region of nts 6954 to 6990 with the high nt substitutions was selected as the anchor and sensor probe sites. Because all of 34 cases had at least one nt substitution (either silent or missense mutation) in the probe sites and especially missense mutations were concentrated in this region. In other word, sequence status of the probe sites is expected to surrogate the genetic alterations of the entire sequence in the ISDR, accounting for the Tm values reflecting mutational status of ISDR. This is actually confirmed by this study. Thus, our PCR–MCA method with the hybridization probe gave promising results enabling us to subcategorize HCV genomes into two major groups; one group with Tm>58.0℃ and the other with Tm<58.0℃ from the viewpoint of IFN efficacy. Compared with sequence results, the former and the latter are roughly equivalent to cases with no aa substitutions and cases with 2 or more aa substitutions, respectively. However, cases having the one aa substitution defined by direct sequencing were distributed between both groups. We can interpret the meaning of this finding, because the Tm value is generated as a comprehensive result based on the total nt substitution number of missense and silent mutations. Accordingly, considering the features of the MCA assay, it is reasonable to designate the high Tm group as wild-type and the low Tm group as mutant-type in a clinical setting. This stratification by Tm, despite the small number of pilot cases, suggests its usefulness in predicting the efficacy of IFN therapy; non-responsive in the high Tm group and responsive in the low Tm group. However, we need a large scale prospective clinical trial to verify the reliability of Tm as a predictive marker, because the validity of the MCA assay in a practical setting has been shown

partially in this study.

Another important point of this MCA assay is to indicate that Tm values after cloning display more clearly the composition profiles of viral variants within HCV quasi-species rather than SSCP. HCV genomes in plasma are generally present as a mixture of heterogeneous genomes, designated quasi-species. To better understand the resistance mechanism against IFN, HCV quasi-species are interesting and instructive, because the pressure of IFN on virus replication could allow the generation and selection of new clones with IFN resistance. Generally, although HCV quasi-species are defined by sequencing or SSCP with targets in the HCV E2 region, 5'NCR etc. regions[23)], the methodology for quasi-species remains problematic, in terms of time and cost. Our study showed that the MCA assay can screen simultaneously and rapidly the degree of genetic complexity of HCV according to the differences in Tm of the ISDR region. To understand the composition of quasi-species precisely, it requires the analysis of a large number of clones. The MCA assay could easily and efficiently pick-up a newly derived clone different from other major clones by comparison of Tm.

In conclusion, this study demonstrates that the MCA assay rapidly and simply provides Tm values reflecting the genetic heterogeneity in the ISDR region of the HCV genome. According to the Tm status provided by this MCA assay, HCV genomes were classified into two types with high and low Tms, corresponding to wild-type ISDR and mutant-type ISDR from the viewpoint of IFN efficacy. Moreover, the MCA assay is convenient and useful to screen newly generated clones by the difference in Tm of the clone relating to the behavior of quasi-species.

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References

1. Asselah T, Bièche I, Paradis V, Bedossa P, Vidaud M, Marcellin P. Genetics, genomics, and proteomics: implications for the diagnosis and the treatment of chronic hepatitis C. Semin Liver Dis 2007;27:13-27.
2. Taura N, Yatsuhashi H, Hamasaki K, Nakao K, Daikoku M, Ueki T, Yano K, Matsumoto T,Ishibashi H,Eguchi K. Increasing hepatitis C virus-associated hepatocellular carcinoma mortality and aging: Long term trends in Japan, Hepatol Res 2006;34:130-4.
3. Shen C, Hu T, Shen L, Gao L, Xie W, Zhang J. Mutations in ISDR of NS5A gene influence interferon efficacy in Chinese patients with chronic hepatitis C virus genotype 1b infection, J Gastroenterol Hepatol 2006;22:1898-1903.
4. Dev A, Patel K, McHutchison JG. New therapies for chronic hepatitis C virus infection. Curr Gastroenterol Rep 2004;6:77-86.
5. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection, N Engl J Med 1996;334:77-81.
6. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Izumi N, Marumo F, Sato C. Comparison of Full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. J Clin Invest 1995;96:224-230.
7. Kmieciak D, Kruszyna L, Migdalski P Lacinski M, Juszczyk J, Trzeciak WH. Mutations within protein kinase R-Binding domain of NS5A protein of hepatitis C virus (HCV) and specificity of HCV antibodies in pretreatment sera of HCV-chronically infected patients and their effect on the result treatment. Jpn J Infect Dis 2006;59:92-99.

8. Sarrazin C, Berg T, Lee JH, Rüster B, Kronenberger B, Roth WK, Zeuzem S. Mutations in the protein kinase-binding domain of the NS5A protein in patients infected with hepatitis C virus type 1a are associated with treatment response, J Infect Dis 2000;181:432-41.
9. Veillon P, Payan C, Guillou-Gullemette HL, Gaudy C, Lunel F. Quasispecies ivolution in NS5A region of hepatitis C virus genotype 1b during interferon or combined interferon-ribavirin therapy, World Vastroenterol 2007;13:1195-1203.
10. Hofmann WP, Zeuzem S, Sarrazin C. Hepatitis C virus-related resistance mechanisms to interferon alpha-based antiviral therapy. J Clin Virol 2005; 32:86-91.
11. Ohno O, Mizokami M, Wu RR, Saleh MG, Ohba K, Orito E, Mukaide M, Williams R, Lau JY. New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. J Clin Microbiol. 1997;35:201-7.
12. Sarrazin C, Mihm U, Herrmann E, Welsch C, Albrecht M, Sarrazin U, Traver S, Lengauer T, Zeuzem S. Clinical significance of in vitro replication-enhancing mutations of the hepatitis C virus (HCV) replicon in patients with chronic HCV infection. J Infect Dis. 2005;192:1710-9.
13. López-Labrador FX, Ampurdanès S, Giménez-Barcons M, Guilera M, Costa J, Jiménez de Anta MT, Sánchez-Tapias JM, Rodés J, Sáiz JC. Relationship of the genomic complexity of hepatitis C virus with liver disease severity and response to interferon in patients with chronic HCV genotype 1b infection [correction of interferon].Hepatology 1999;29:897-903.
14. Pawlotsky JM, Germanidis G, Neumann AU, Pellerin M, Frainais PO, Dhumeaux D. Interferon resistance of hepatitis C virus genotype 1b: relationship to nonstructural 5A gene quasispecies mutations.J Virol. 1998;72:2795-805.

15. N Kato, M Hijikata, Y Ootsuyama, M Nakagawa, S Ohkoshi, T Sugimura, and K Shimotohno. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci U S A 1990;87:9524-9528.
16. Mori S, Sugahara K, Uemura A, Akamatsu N, Tutsumi R, Kuroki T, Hirakata Y, Atogami S, Hasegawa H, Yamada Y, Kamihira S. Rapid, Simple, and Accurate Detection of K-ras Mutations From Body Fluids Using Real-Time PCR and DNA Melting Curve Analysis. Labmedicine 2006;37:286-289.
17. Alter MJ, Margolis HS, Krawczynski K, Judson FN, Mares A, Alexander WJ, Hu PY, Miller JK, Gerber MA, Sampliner RE, et al. The natural history of community-acquired hepatitis C in the United States. The Sentinel Counties Chronic non-A, non-B Hepatitis Study Team. N Engl J Med 1992; 327:1899-905.
18. Chevaliez S, Pawlotsky JM. Hepatitis C virus: virology, diagnosis and management of antiviral therapy. World J Gastroenterol 2007;13:2461-6.
19. Gerotto M, Sullivan DG, Polyak SJ, Chemello L, Cacalletto L, Pontisso P, Alberti A, Gretch DR. Effect of retreatment with interferon alone or interferon plus ribavirin on hepatitis C virus quasispecies diversification in nonresponder patients with chronic hepatitis C. J Virol 1999;73:7241-7247.
20. Bowden DS, Berzsenyi MD. Chronic hepatitis C virus infection: genotyping and its clinical role. Future Microbiol 2006;1:103-12.
21. Zhou L, Wang L, Palais R, Pryor R, Wittwer CT. High-resolution DNA melting analysis for simultaneous mutation scanning and genotyping in solution. Clin Chem 2005;51:1770-7.
22. Lyon E. Mutation detection using fluorescent hybridization probes and melting curve analysis. Expert Rev Mol Diagn 2001;1:92-101.
23. Schvoerer E, Soulier E, Royer C, Renaudin AC, Thumann C, Fafi-Kremer S, Brignon N, Doridot S, Meyer N, Pinson P, Ellero B, Woehl-Jaegle ML, Meyer

C, Wolf P, Zachary P, Baumert T, Stoll-Keller F. Early evolution of hepatitis C virus (HCV) quasispecies after liver transplant for HCV-related disease.J Infect Dis 2007;196:528-536.

Legends

Figure 1. The frequency of nucleotide (nt) substitution (open column; silent mutations, solid column; missense mutations) in the ISDR region from nt 6954 to 7073.

Although silent mutations are widely distributed, missense mutations are mainly concentrated to the region of nts 6960 to 6990 corresponding to codons 2211 to 2221. At least one nt substitution was detected within the region corresponding to the probe site in all cases.

Figure 2. A correlation between melting temperature and the quantity of nt Substitutions

A: The number of nt substitutions is inversely correlated to melting temperature ($r=-0.689$, $p<0.01$). There is a tendency to form two clusters: one with high Tm and low number of nt substitutions (dot line circle), and the other one with low Tm and high number of nt substitutions (solid line circle).

B: The number of missense mutation (aa substitution) is more significantly correlated to Tm ($r=-0.617$, $p<0.01$). There appears to exist a boundary (dot line) in Tm at 58.0℃, discriminating into two clusters; one cluster with high Tm and no or one aa substitution, and the other one with low Tm and 2 or more aa substitutions.

Figure 3. The difference of IFN efficacy in cases classified by Tm status (more or less than 58.0℃) by monitoring HCV viral load after interferon treatment in patients infected with HCV (solid broken line; HCV Ag

Detection limit of <=20 fmol/L)

A: Cases with Tm=59.3℃, 62.5℃, and 64.8℃. All of 3 cases were transiently responsive, but not sustained; namely non-sustained virological responders.

B: Cases with Tm=56.1℃ and 43.73℃. These 2 cases with low Tm values showed sustained viral response.

Figure 4. PCR-SSCP analysis for the NS5A 2209-2248 region, indicating the presence of the heterogeneity in the ISDR sequence in lanes 1, 3, 4, 7, 8, 11, 12, 13, 14, 15, 18, and 20. M; marker

Figure 5. Melting curves (upper) and dot plots (lower) of Tm after cloning of each isolate prior to treatment, showing that the HCV genome is as a mixture of clones with subtle variation in Tm relating to HCV quasi-species:

Solid circle (arrow head); Tm of amplicons,

Other triangles ; Tm of clones

The MCA assay provides subtly different Tm values between clones reflecting the degree of the genetic heterogeneity of 3 HCV samples. In patient No. 17, they consist of two major clones with Tm=50.8℃ and 57.8℃, and several scattered clones; in patient No 28, of one major clone with Tm=64.0℃ and rare minor clones with Tm around 55.0℃; and in patient No 37, of one major clone with Tm=44.0℃ and other minor clones.

Figure 6. Observation of changes in the composition of clones by the MCA assay during IFN therapy;

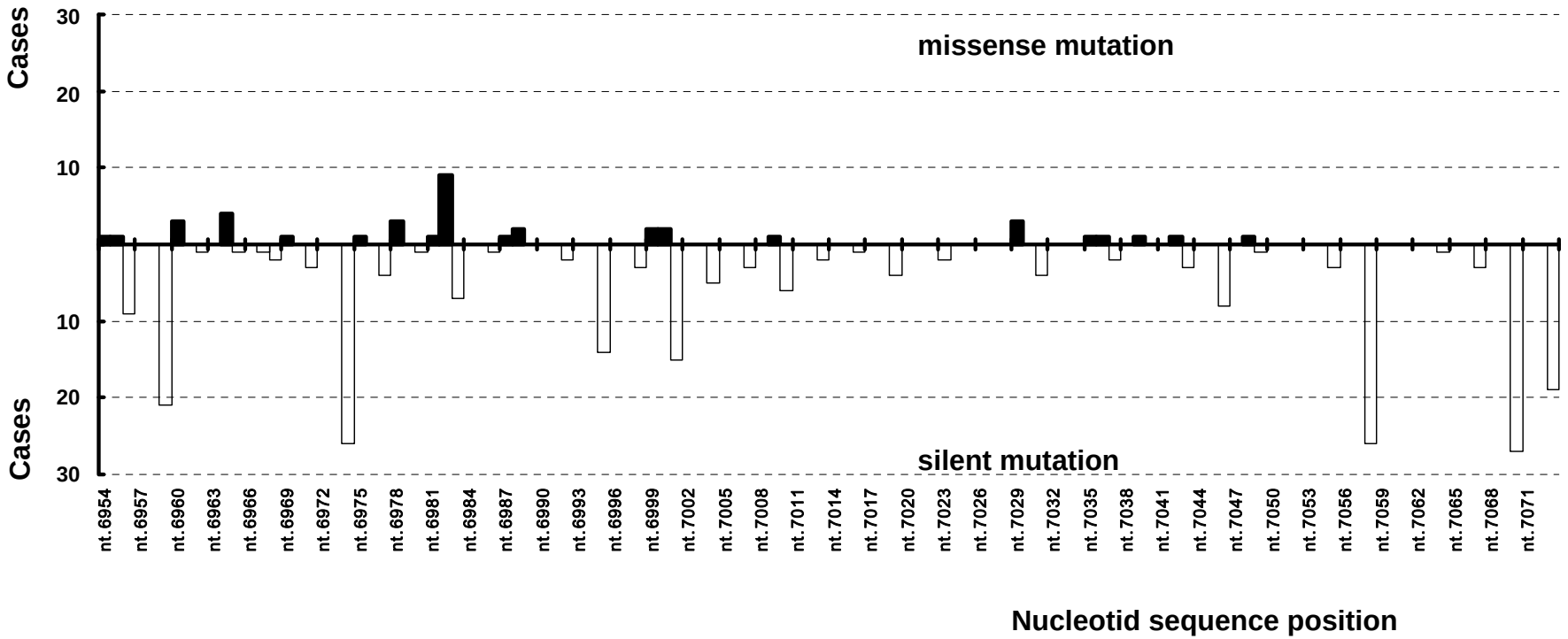
(A); Changes in HCV viral load after IFN therapy

(B); Sequential observation of clones at 3 clinical points by MCA assay

Although the Tm value was 61.2℃ in isolate 1, 60.7℃ in isolate

2, and 60.9℃ in isolate 3, the Tm values after cloning from each isolate showed that many different clones exist, characterized by subtly different Tm values, relating to quasi-species. In particular, the disappearance of clones with low Tm (dot line circle in Figure B) in isolate 2 is noteworthy, which reappear again in isolate 3. The major clones with high Tm remained unchanged during the entire clinical course.

Figure 1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30
20
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missense mutation
10
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Cases
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silent mutation
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nt.7044
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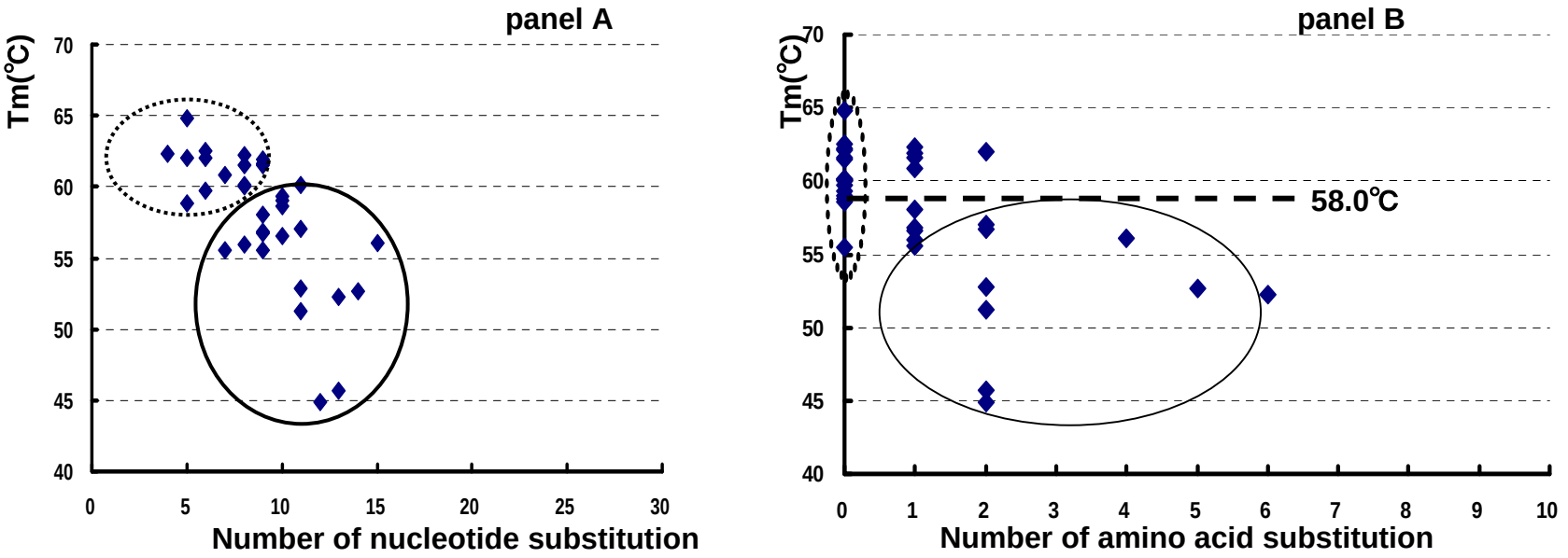
Nucleotid sequence position

Figure 2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Tm(℃)
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Number of nucleotide substitution
panel B
Tm(℃)
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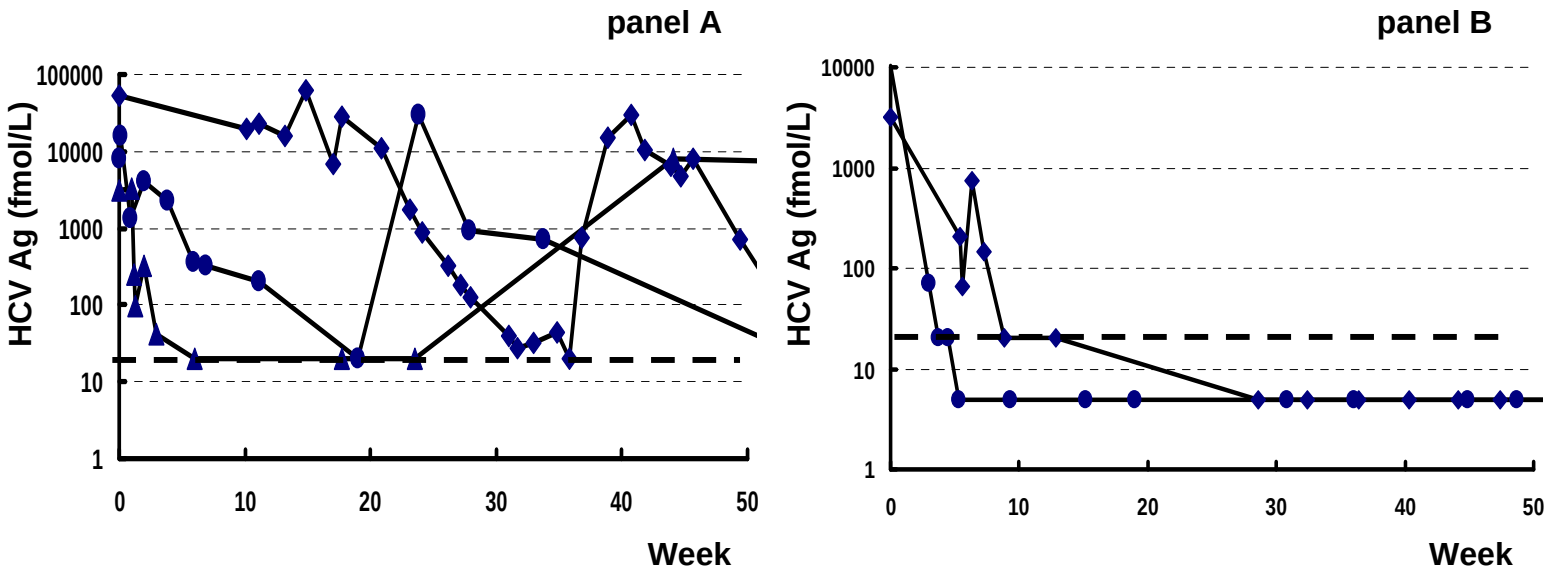
Number of amino acid substitution

Figure 3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HCV Ag (fmol/L)
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Figure 4

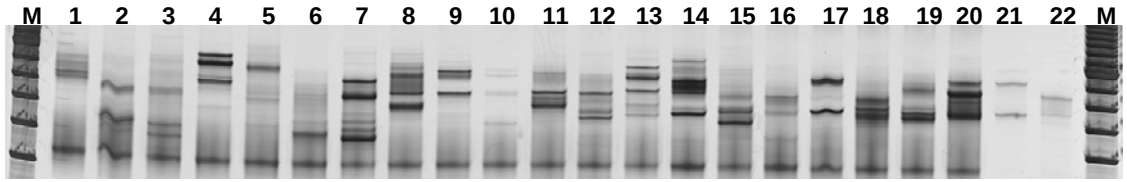

Figure 5

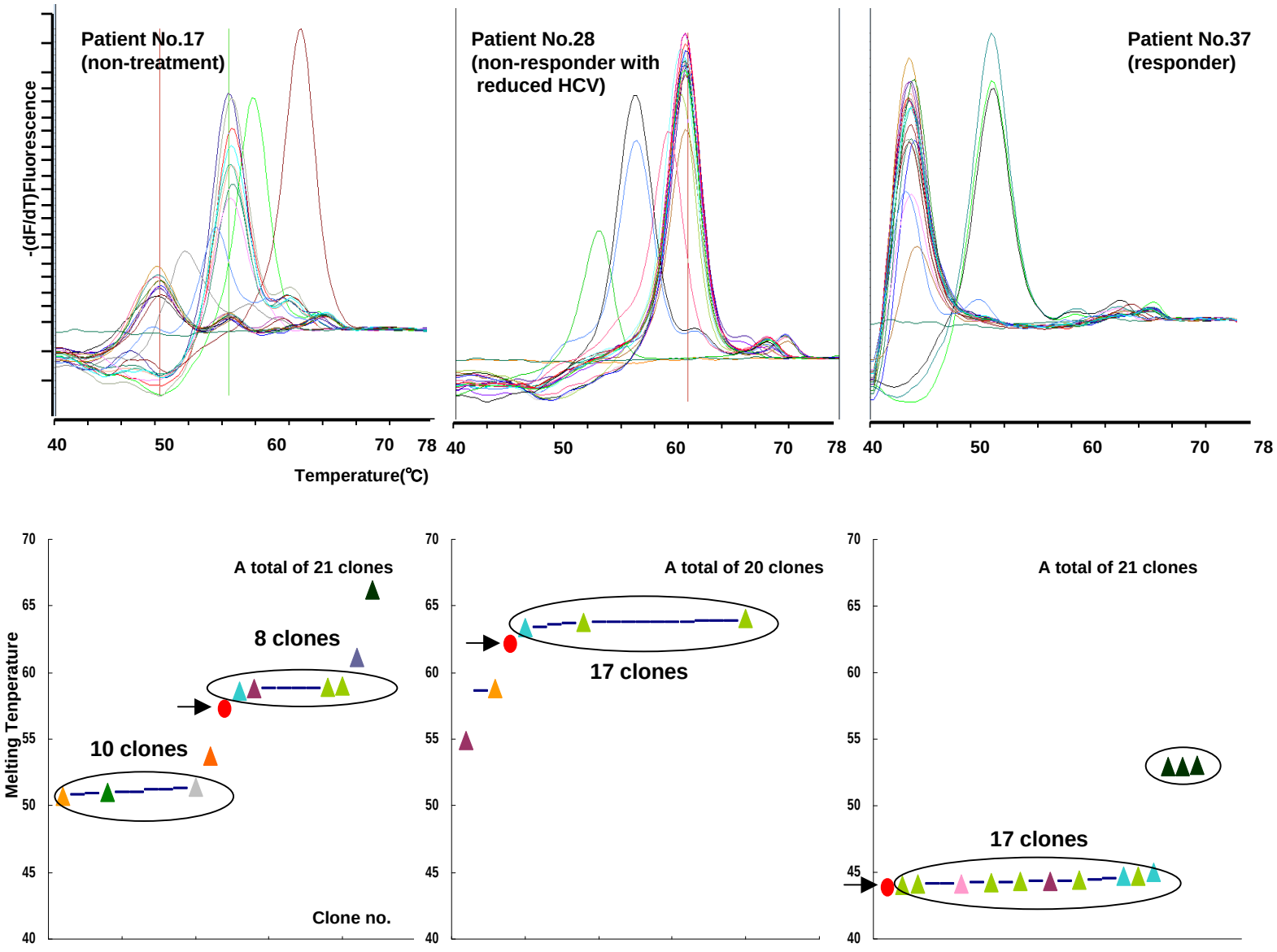
Patient No.17
(non-treatment)
Patient No.28
(non-responder with
reduced HCV)
Patient No.37
(responder)
-(dF/dT)Fluorescence
40
50
60
70
78
Temperature(℃)
Melting Tenperature
A total of 21 clones
8 clones
10 clones
Clone no.
A total of 20 clones
17 clones
A total of 21 clones
17 clones

Figure 6

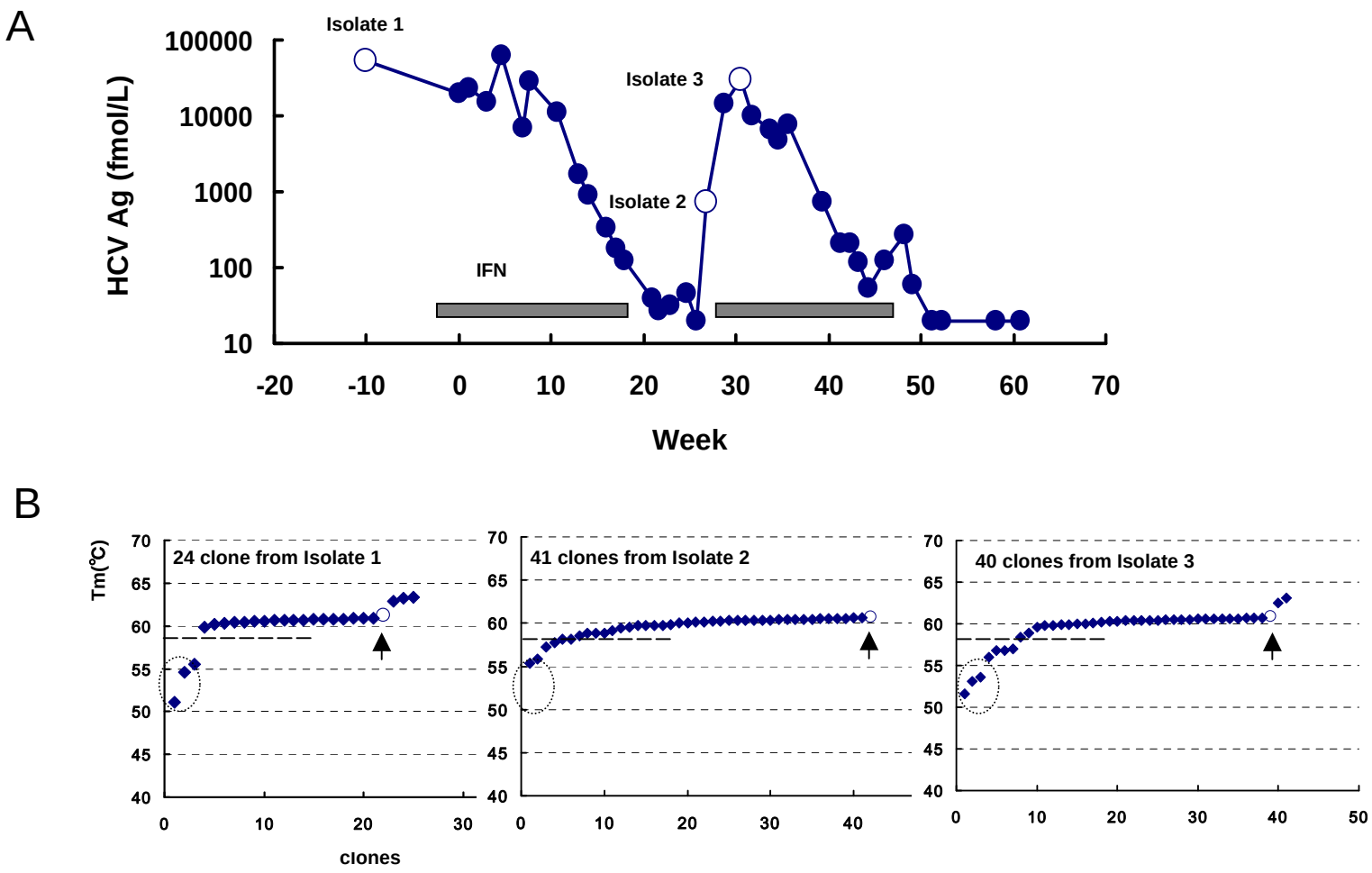
A
Isolate 1
Isolate 3
Isolate 2
IFN
HCV Ag (fmol/L)
100000
10000
1000
100
10
-20
-10
0
10
20
30
40
50
60
70
Week
B
24 clone from Isolate 1
41 clones from Isolate 2
40 clones from Isolate 3
Tm(℃)
70
65
60
55
50
45
40
0
10
20
30
40
50
clones

Table 1 Characteristics of patients enrolled in the study.

Case No.	Patient No.	Age	Sex	HCV genotype	Viral Load (KIU/ml)	HCV Ag (fmol/L)	disease	treatment	Response
1	No.01	69	M	1b	313	ND	CH	NT	
2	No.02	63	M	1b	1650	ND	AC	NT	
3	No.04	35	M	1b	2140	ND	CH	NT	
4	No.05	44	F	1b	1880	ND	CH	NT	
5	No.06	63	F	1b	2430	ND	CH	NT	
6	No.08	41	F	1b	638	ND	AC	NT	
7	No.09	68	F	1b	2810	ND	AC	NT	
8	No.10	65	F	1b	7980	ND	CH	NT	
9	No.11	66	F	1b	2490	ND	CH	NT	
10	No.12	74	M	1b	514	ND	CH	NT	
11	No.13	65	F	1b	2510	ND	HCC	NT	
12	No.15	55	F	1b	>5000	52000	LC	IFN + R	NR
13	No.16	55	M	1b	4500	19622	CH	NT	
14	No.17	60	M	1b	2100	3010	CH	NT	
15	No.18	72	M	1b	ND	380	CH	IFN + R	PR
16	No.19	55	F	1b	ND	15600	CH	IFN + R	NR
17	No.20	55	F	1b	2800	4420	CH	IFN + R	NR
18	No.21	59	M	1b	1200	1560	CH	NT	
19	No.23	54	F	1b	>5000	62200	LC	IFN + R	PR
20	No.25	33	F	1b	ND	1280	CH	NT	
21	No.27	73	F	1b	490	ND	CH	NT	
22	No.28	62	F	1b	>5000	17800	LC	IFN + R	NR
23	No.29	65	F	1b	ND	11900	CH	NT	
24	No.30	52	F	1b	ND	5480	CH	IFN + R	PR
25	No.31	70	F	1b	ND	9280	CH	NT	
26	No.32	46	M	1b	ND	11000	CH	NT	
27	No.33	62	F	1b	ND	3060	HCC	IFN + R	NR
28	No.34	57	M	1b	1200	3210	HCC	IFN + R	SVR
29	No.36	70	F	1b	ND	7390	AC	NT	
30	No.37	71	M	1b	ND	10500	CH	IFN + R	SVR
31	No.38	56	F	1b	ND	3070	CH	IFN + R	NR
32	No.39	65	M	1b	ND	5210	CH	IFN + R	PR
33	No.40	58	M	1b	ND	2050	LC	IFN + R	NR
34	No.41	47	M	1b	ND	11200	CH	IFN + R	PR

Out of 34 cases, 14 underwent IFNqribavirin therapy, but the remaining cases did not undergo such therapy because of severe complications, such as cancers, pneumonias, etc. ND, not done; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; AC, asymptomatic carrier; IFNqR, interferon (IFN) and ribavirin (R); NT, non-treatment; NR, nonresponse; PR, partial response; SVR, sustained virological response.

Table 2. Sequence profile of primers, and sensor and anchor hybridization probes designed for the present study

Forward primer	5’-ACCGACCCCTCTCATATTAC-3’
Reverse primer	5’-GATCGAAAGAGTACAGGATTAC-3’
Anchor probe (3’labelled FITC)	5’-GCCAGCTCTTCAGCTAGCCAGTTGTCTGCGCCTTCTTTGAAG-3’
Sensor probe (5’labelled LCRed640)	5’-CGACATGTACTACCCATCATGACT-3’-P

Table 3

Characteristics of the mutation profile of ISDR sequence and viral load in two groups classified by the Tm=58.0℃ boundary

	average		
	nucleotide	amino acid	HCV Ag
HCV with high Tm	7.7 ±2.0 *	0.4±0.6*	15063±18952
HCV with low Tm	10.9±2.3 *	2.1±1.7*	6291±4097

*; statistically significant ($p<0.01$)