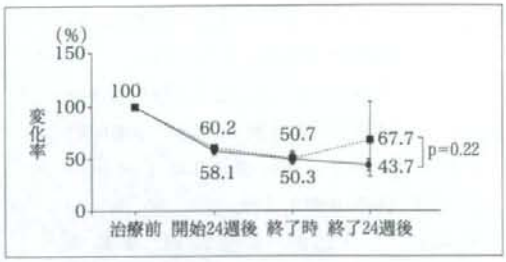

図3 PEG-IFNα2b+リバビリン併用例のHCVコア抗体価変化率
実線がSVR例，破線が非SVR例の変化を示す．

療法を施行し，効果判定の可能な26例を対象とした．男性14例，女性12例で，年齢は50.7±10.9歳．SVRが19例で，非SVR例は7例である．HCVコア抗体は，治療開始前/開始24週間後/治療終了時/終了6カ月後の4ポイントで測定した．

治療開始前のHCVコア抗体価を100%とした場合の平均変化率は，SVR例では100/58.1/50.3/43.7%，非SVR例では100/60.2/50.7/67.7%であった(図3)．開始前値より50%以上の抗体価低下を認めた例は，治療開始24週の時点では，SVR例の7例(36.8%)が非SVR例の1例(14.3%)よりも高率の傾向を認めたが，治療終了時では，それぞれ11例(57.9%)，4例(57.1%)で，治療効果の予測に有用な結果は得られなかった．

IV. 考　察

IFN治療終了後のHCVコア抗体測定はHCV排除の確認目的として有用である．治療開始前の抗体価を基準とした抗体価変化率も，治療の反応が良好な例で高率の傾向を示した．24週間治療例の有効性と抗体価変化率との検討結果から，治療期間が長期化した場合に，治療終了前に抗体価の変化から効果予測へ結びつく可能性も期待された．しかし，残念ながら，難治例のIFN標準治療期間が48週間と長期化した現治療でも，48週間の治療期間内の抗体価測定からは，治療効果判定に有用な結果は得られなかった．とくに，48週間の治療終了時点では，SVR群，非SVR群の両群において，抗体価の平均低下率も50%以上低下例の比率も同等であった．

初期に検討した24週間治療例での著効例に比べて，48週間治療例は難治である症例も含まれ，肝臓内からHCVが完全に排除されてからの期間が短期である可能性も考えられる．著効例においては，治療後の時間の経過とともにHCV抗体価が低下する傾向が示されており[2]，どの程度の期間経過が判定に妥当であるか，あるいは，より長期間の治療を行った場合の評価などが，今後の検討課題であると思われる．

まとめ

肝臓内からHCVが完全排除された場合にHCVに対する抗体価は経時的に低下する．HCV抗体価の測定は，核酸測定に比べて安定した結果が得られるが，現在の標準治療においては，治療期間中に効果判定の予測に応用することは困難であった．

文　献

1) 松嶋　喬，髭　修平，狩野吉康，他：効果判定のマーカー．臨床医　20；1508-1511，1994
2) 石川和克，小山富子：スクリーニング検査法としての市販HCV抗体測定法の特徴．日本臨牀　62(増刊号7)；199-207，2004

Original Article

Quantification of lamivudine-resistant hepatitis B virus mutants by type-specific TaqMan minor groove binder probe assay in patients with chronic hepatitis B

Shigeru Yoshida[1], Shuhei Hige[3], Miyuki Yoshida[2], Naoki Yamashita[2], Shin-ichi Fujisawa[2], Kaori Sato[2], Tadashiro Kitamura[2], Masaharu Nishimura[4], Makoto Chuma[3], Masahiro Asaka[3] and Hitoshi Chiba[1]

[1]Department of Health Sciences, Hokkaido University School of Medicine, North-12, West-5, Sapporo 060-0812; [2]Department of Clinical Laboratory, Hokkaido University Hospital, Sapporo 060-8648; [3]Gastroenterology and Hematology Section, Department of Internal Medicine, Hokkaido University Graduate School of Medicine, Sapporo 060-8638; [4]First Department of Medicine, Hokkaido University School of Medicine, Sapporo 060-8638, Japan

Corresponding author: Dr Shigeru Yoshida. Email: shiyoshi@med.hokudai.ac.jp

Abstract

Background: Lamivudine (LAM)-resistant hepatitis B virus (HBV) with mutations in the polymerase region frequently appears after long-term use of LAM. Several methods allowing detection of mutant strains (YIDD, YVDD) have been reported, but they have no quantitative characteristics. In this study, we explored a unique approach for quantification of each mutant strain.

Methods: A method for detection and quantification of wild and mutant strains was developed using realtime polymerase chain reaction and type-specific minor groove binder (MGB) probes, and tested in patients with chronic hepatitis B before and after additive treatment with adefovir dipivoxil (ADV).

Results: A good correlation was confirmed in HBV DNA quantity obtained between the YMDD-specific MBG probe assay and Amplicor HBV Monitor assay results ($P < 0.001$), linear between 3 and 9 log copies/mL serum. Of 109 samples from patients with chronic hepatitis B tested by both these assays and conventional direct sequencing, 90 (88.2%) showed identical results. The assays successfully detected and quantified a single type of mutant in three of four patients with additive ADV treatment, and also two coexisting mutant types (YIDD and YVDD) in the remaining patient.

Conclusions: Our specific and sensitive method for detection and quantification of HBV DNA with the wild-type YMDD motif and its two mutant forms (YIDD and YVDD) appears to be clinically useful, especially in patients with multiple mutant HBV infections.

Ann Clin Biochem 2008; **45**: 59–64. DOI: 10.1258/acb.2007.006219

Introduction

Lamivudine (LAM) was initially synthesized as an anti-human immunodeficiency virus (HIV) drug and was later found to inhibit hepatitis B virus (HBV) proliferation as well as to decrease copy number of HBV DNA and to improve liver function in hepatitis patients. However, in many cases HBV gains tolerance to LAM after long-term use,[1–3] requiring the use of interferon in addition to conventional hepato-protective drugs.[4] Recently, another novel nucleoside analogue, adefovir dipivoxil (ADV) was reported to have strong antiviral effects on LAM-resistant mutants.[5–9] The active centre of reverse transcriptase of LAM-resistant HBV has a lower affinity for LAM than that of wild-type HBV,[10] the most common mutations in the YMDD motifs in association with tolerance to LAM being YIDD (rtM204I) and YVDD (rtM204V) affecting the tyrosine-methionine-aspartate-aspartate (YMDD) motif (amino acid 203–amino acid 206) in domain C of the polymerase region.[10–12] HBV infection can occur with not only one, but also a mixture of viruses; but the detection and quantification of an individual HBV type is difficult, especially when the viral load differs greatly among the infected virus types.[13,14] In this study, we have developed a unique approach to measuring mutant HBV by realtime polymerase chain reaction (PCR) using type-specific minor groove binder (MGB) probes, and applied the method to patients with chronic hepatitis B receiving additive treatment with ADV.

Methods

Patients

Four patients with chronic hepatitis B who had been treated with LAM and were later found to have breakthrough hepatitis associated with the appearance of mutated HBV were enrolled in this study. The patients initially received 100 mg of LAM every day, and then were additionally administered 10 mg of ADV once daily. At the beginning of the combination therapy of LAM and ADV, the YMDD motif mutation was found in all four cases; two also featured YIDD and the other two YVDD. The average duration of the combination therapy was 149 days (112–189 days), and the average HBV load at the beginning of the combination therapy was 7.44 log copies/mL (6.74–8.73 log copies/mL). Informed consent for participation in this study was obtained from all patients.

Extraction of hepatitis B virus DNA

Viral DNA was extracted from 200 μL of serum using a QIAamp DNA Blood Mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions.

Quantification of hepatitis B virus DNA

To measure the quantity of HBV DNA, AMPLICOR™ HBV MONITOR (Roche Diagnostics, Tokyo, Japan) was used. HBV DNA in the samples that exhibited high viral load beyond the upper limit of detection (7.6 log copies/mL) were re-examined by the 100-fold dilution method, which was validated previously.[15] The overall range of our measurements spanned between 2.6 and 9.6 log copies/mL.

Type-specific minor groove binder probe assay

To measure the quantity of the wild-type strain (YMDD) and the two mutant types (YIDD and YVDD), we developed a type-specific TaqMan MGB probe assay. For this purpose, three reaction mixtures, each containing one type-specific probe, were prepared and the quantity of HBV DNA was measured by a realtime PCR method using an external standard. One mixture contained 1× Platinum Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA) (0.75 U of Platinum *Taq* polymerase, 20 mmol/L of Tris-HCl [pH 8.4], 50 mmol/L of KCl, 200 μmol/L of each dNTP), 0.4 μmol/L of a sense primer (HBV-MGB2-S: 5′-TCCTATGGGAGTGGGCCTC-3′), 0.4 μmol/L of an anti-sense primer (HBV-MGB-AS: 5′-GTACAGACTTGGCCCCC AAWAC-3′), 0.4 μmol/L of a type-specific TaqMan MGB probe (YMDD: 5′-FAM CTTTCAGTTATATGGATGAT MGB-3′, YIDD: 5′-FAM CTTTCAGTTATATTGATGAT MGB-3′, YVDD: 5′-FAM TTTCAGTTATGTGGATGAT MGB-3′) and 4.0 mmol/L $MgCl_2$. The total volume was adjusted to 25 μL by adding 5 μL of a DNA sample (or external standard) and DNase- and RNase-free water. Realtime PCR was performed using a 7500 RealTime PCR System (Applied Biosystems, Foster City, CA, USA). The PCR conditions were as follows: 50°C for 2 min and 95°C for 2 min for the initial period and then 95°C for 25 s and 62°C for 60 s for 45 cycles. Regarding analytical conditions for the 'baseline', three cycles for 'start' and 15 cycles for 'stop' were used. The threshold concentration was designated as 0.005 for both YMDD and YIDD and 0.02 for YVDD. HBV DNA concentrations were presented as log copy numbers in 1 mL of serum (log copies/mL).

External standard

In order to construct three external standards for the three type-specific reactions, PCR products from each of the three strains were obtained using the primer sets of HBV-MGB-2 and HBV-MGB-AS, and then inserted into the pGEM T Easy Vector (Promega, Madison, WI, USA). Each plasmid was linearized by *Sac*I digestion and copy numbers were assessed by absorption spectrophotometry and adjusted to 2×10^5, 2×10^3, 2×10^1 and 2×10^0 copies/mL. Five microlitres of each concentration was added to the reaction mixture as described above.

Amplification of the hepatitis B virus DNA polymerase region by polymerase chain reaction

The YMDD motif was analysed by direct sequencing after amplification of the HBV DNA polymerase region, performed in 25 μL of a reaction mixture containing 1× PCR Master Mix (Promega) (50 U/mL of *Taq* DNA polymerase in a supplied reaction buffer [pH 8.5], 400 μmol/L of each dNTP, 3 mmol/L $MgCl_2$), 0.4 μmol/L of a sense primer (HBV-1-S: 5′-TGGCTATCGCTGGATGTGTCT-3′), 0.4 μmol/L of an anti-sense primer (HBV-3-AS: 5′-CGGGC AACGGGGTAAAGG-3′) and 5 μL of sample DNA. The reaction was performed with a GeneAmp™ 9700 (Applied Biosystems) at 94°C for 2 min for the initial incubation, 94°C for 30 s for denaturing, 57°C for 30 s for annealing, 72°C for 30 s for extension for 40 cycles and a final extension step of 72°C for 7 min. The PCR product of 794 bp was confirmed on a 2% agarose gel.

Amplification of the hepatitis B virus DNA polymerase region by nested polymerase chain reaction

When any PCR product was not observed by the above method, nested PCR was performed using 1 μL of the first PCR product with a sense primer (HBV-5-S: 5′-CCCTC WGTTGCTGTACAAAACCT-3′) and an anti-sense primer (HBV-1-AS: 5′-AAAGGCATTAAGGCAGGATA-3′) under the same conditions as for the first round of PCR. The nested PCR product of 505 bp was confirmed by agarose gel electrophoresis.

Sequence analysis of the YMDD motif

PCR products were purified using a QIAquick PCR purification kit (QIAGEN GmbH) before sequence analysis using a BigDye Terminator cycle sequence kit version 3.1 (Applied Biosystems) with HBV-5-S as the sequence primer. After the reaction, the sample was treated with a DyeEX 2.0 Spin kit (QIAGEN GmbH), and was electrophoresed in an ABI PRISM genetic analyzer 310 (Applied

Biosystems). The amino acid sequence was determined with DNA sequencing analysis software version 3.0 (Applied Biosystems). When mixed bases were read on the same position, the nucleotide sequences were determined according to all visible peaks and translated into amino acids.

Results

Linearity

The linearity of HBV DNA quantification by the present method was confirmed by measuring serial dilution of plasmids carrying PCR products of YMDD, YIDD and YVDD strains. The dilution curve was linear between 1.0 and 7.0 log copies for each strain (Figure 1).

Specificity

In order to study the specificity of the YMDD-specific HBV DNA quantification, the three reaction mixtures, each containing one type-specific probe, were added with DNA (6.0 log copies) from the YMDD strain, and measurement was conducted as described above. When the combination of probe and HBV DNA type was matched, an increase in delta-normalized reporter (ΔRn) beyond 0.05 was observed. In the case of a mismatched probe, the intensity was below 0.02 (Figure 2). For YIDD and YVDD strains, the same procedures were performed, and comparable results were obtained.

Reproducibility

When HBV DNA quantification was carried out 10 times for the same sample, the coefficients of variation were 1.0% for YMDD, 2.2% for YIDD and 1.9% for YVDD.

Comparison with a commercial kit

The present method was compared with a commercial PCR kit (AMPLICOR™ HBV MONITOR) using serum samples from patients (n=14) with chronic hepatitis B who were not treated with LAM. Because all patients had YMDD

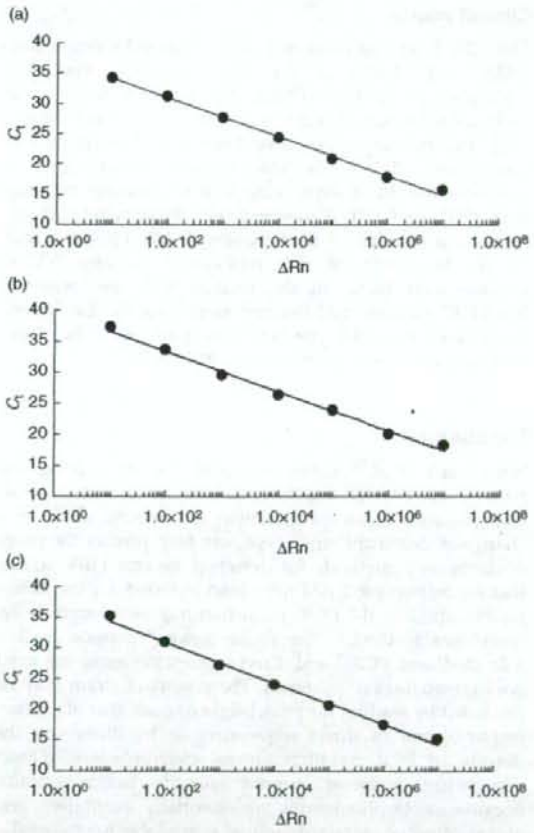

Figure 1 Dilution curves by titration of linearized plasmids. Type-specific TaqMan minor groove binder probe assays using a plasmid corresponding to YMDD motif (a), YIDD (b) and YVDD (c)

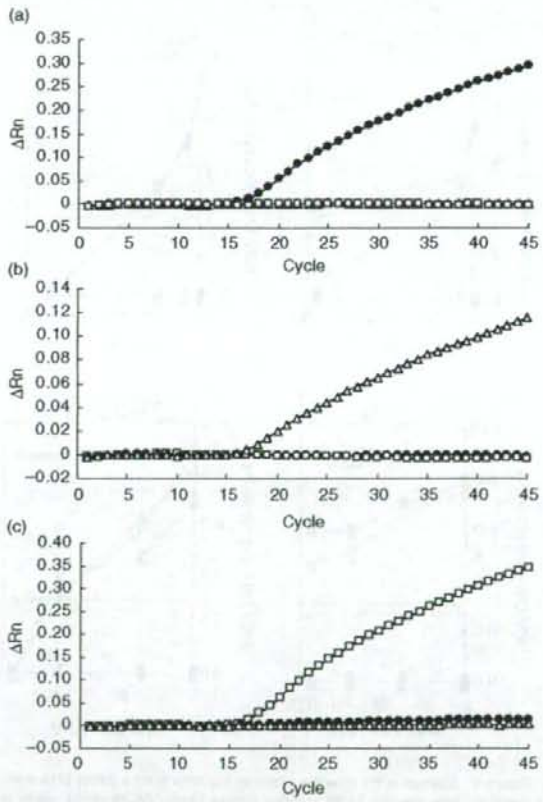

Figure 2 Specificity of YMDD (a), YIDD (b) and YVDD (c) specific TaqMan minor groove binder probe assays. Symbols represent linearized plasmids corresponding to YMDD (●), YIDD (△) and YVDD (□)

Table 1 A comparison of hepatitis B virus mutant types obtained by type-specific TaqMan minor groove binder (MGB) probe assay and direct sequencing

	TaqMan MGB probe assay					
Direct sequencing	YMDD	YIDD	YVDD	Y[M/I]DD	Y[M/I/V]DD	ND
YMDD	39			1		
YIDD		34		1		
YVDD			14			
Y[M/I]DD	1	1		3		
Y[M/I/V]DD		2	2		4	
ND						7

ND, not detected

strains but no YIDD or YVDD strains, only values for the YMDD strain were compared. A strong correlation was found between the two assays ($y = 0.972x + 0.214$, $r = 0.98$, $P < 0.001$).

Sensitivity

Two of the three linearized plasmid solutions, containing sequences corresponding to YMDD, YIDD or YVDD, were mixed at mixing ratios of 100:100 (7.6 log copies each), 100:10, 100:1, 10:100 and 1:100. The resulting three sets of plasmid mixtures (YMDD vs. YIDD, YMDD vs. YVDD and YIDD vs. YVDD) were then measured. The plasmid corresponding to YMDD (or YIDD) was detectable and measurable down to 1% of YIDD and YVDD (or YMDD and YVDD). The plasmid corresponding to YVDD was detectable to 1% of YMDD and YIDD, but measurable only at 10%.

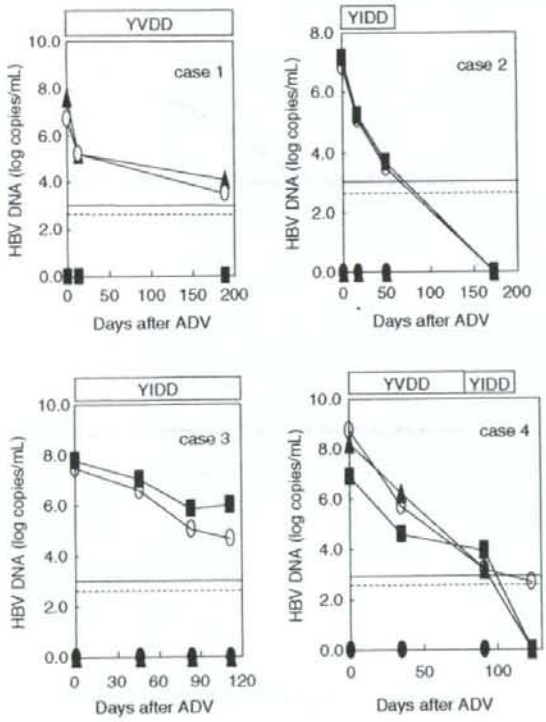

Figure 3 Change in the quantity of serum hepatitis B virus (HBV) DNA monitored by type-specific TaqMan minor groove binder (MGB) probe assay in patients treated with adefovir dipivoxil. Horizontal lines represent the detection limits of type-specific TaqMan MGB probe assay (– – –) and Amplicor HBV Monitor (—). Symbols represent YMDD DNA (●), YIDD DNA (■), YVDD (▲) and Amplicor HBV Monitor (○)

Comparison with conventional direct sequencing

Both the MGB probe assay and direct sequencing were performed for 109 chronic hepatitis B patients on LAM therapy (Table 1). HBV DNA was detected in 102 (93.6%) of the samples, and identical results were obtained for 90 (88.2%) patients with both methods. In the other 12 samples, direct sequencing resulted in overestimation in 10 cases (9.8%) and underestimation in two (2.0%), in comparison with the type-specific MGB probe assay.

Clinical course

The HBV load was measured after combined therapy with LAM and ADV in the four patients who had LAM-resistant mutant HBV(s) (Figure 3). In three (cases 1–3), a single mutant strain was detected by direct sequencing, and the same type was detected and quantified by type-specific MGB probe assay. In the other case (case 4), suggested by direct sequencing to have multiple mutants, both YIDD and YVDD were individually detected and successfully quantified by the type-specific MGB probe assays. At the beginning of the combination therapy, YVDD mutants were found by this method to be predominant, but YIDD mutants had become more common by day 91. In all cases, the wild-type HBV remained below the detection limit at all time points during the study.

Discussion

Natsuizaka *et al.*[16] earlier reported that monitoring the pattern of YMDD motif mutations is useful because changes, such as an accumulation of a specific strain or a change of dominant strain type, can help predict the prognosis. Several methods for detecting mutant HBV strains that are resistant to LAM have been reported. Of the different possibilities, the PCR–restriction fragment length polymorphism method,[17] line probe assay,[18] peptide nucleic acid-mediated PCR[19] and direct sequencing assay are used only for qualitative purposes. The dominant strain may be predicted by reading the peak height or peak area of an electropherogram of direct sequencing or by observing the density of PCR products on gel electrophoresis. Direct sequencing, however, cannot quantify mutant strains because electropherograms are essentially qualitative, and also because no standard method is available for the evaluation of mixed bases. Amino acid sequences cannot be identified when mixed bases are observed in the same codon.

In our present study, quantification of each strain of HBV DNA proved possible with realtime PCR using a unique TaqMan MGB probe for each mutant strain. TaqMan MGB probe was constructed with a fluorescent reporter dye at the 5′ end and with a non-fluorescent quencher and MGB at the 3′ end. A non-fluorescent quencher is thought to contribute to lower noise in comparison with a fluorescent quencher. The MGB in the probe, on the other hand, elevates the melting temperature (T_m) through binding to the double-stranded DNA composed of the probe oligonucleotide and the single-stranded target DNA. For this reason, the MGB probe gives a significant change in T_m even when only a single mismatch exists in the target, and thus can be used for single nucleotide polymorphism analysis.[20] As the YIDD and YVDD motifs were both generated from the YMDD motif by single base mutations (M204I: ATG→ATT, M204V: ATG→GTG), this property of the MGB probe was advantageous for our assay. Actually, when the TaqMan MGB probe was changed from a regular one in our experiment, we confirmed that the T_m value was elevated from 40.4–46.2°C to 63.1–65.3°C using the MGB probe. The latter temperature zone meets the requirement of realtime PCR.

Punia *et al.*[21] reported a method for quantification of LAM-resistant HBV DNA by realtime amplification refractory mutation system PCR. In comparison with their method, our approach provides a better absolute detection limit: 3 vs. 4 log copies/mL of serum. However, regarding relative detection limits, they reported 0.01% of total HBV for YIDD, while ours were 1.0% for YMDD and YIDD, and 10% for YVDD. A possible explanation for the poor relative detection limit of our method is that the specific binding of the probe to the target strain suffers from competition by other strains that are inferior in affinity for the probe but present at a larger copy number than the target. Another realtime PCR assay based on melting curve analysis using fluorescent biprobe hybridization has been reported.[22] This assay gives the ratio of copy numbers of HBV mutants to the total HBV, but not the concentration of HBV mutants. As T_m is influenced by any type of mismatch under the probe, a mismatch adjacent to the YMDD sequence can affect the measurement.

Using conventional sequencing, we overestimated the number of strains in 9.8% of the studied samples and underestimated in 2.0%, when compared with our proposed method. The overestimated samples possessed mixed bases within a codon, presumably due to the presence of multiple mutants or to a simple drift of the baseline in electropherograms. The underestimation with our sequencing method might possibly be due to its low sensitivity relative to our proposed method.

When we applied our method to patients with chronic hepatitis B who received additional ADV after the emergence of LAM-resistant mutants, we could successfully quantify the mutant types in cases 1–3 in good accordance with the results of AMPLICOR™ HBV MONITOR and direct sequencing. The merit of our method, however, was confirmed best in case 4, whose dominant HBV was determined as a YVDD strain by direct sequencing at the start of ADV administration; but was found by our method to shift to YIDD and YVDD strains in variable proportions during the course of treatment. Briefly, the YVDD was about 1 log larger than the YIDD at the beginning of ADV administration; while on day 90, the YIDD was about 1 log larger than the YVDD. This change in the strain type during the treatment is of interest because it is reported that tolerance to LAM differs depending on the mutant type.[23] If YVDD were more susceptible to ADV than YIDD, the observed change in the strain type in case 4 would be well explained. A previous report using molecular modelling which provided evidence that the mutant type is more susceptible to ADV than the wild type supports this possibility.[24]

In conclusion, the present method can provide type-specific and quantitative information on multiple HBV strains quickly and reliably. Because drug resistance is a major hazard in anti-HBV therapy, this method might be useful for clinical purposes to monitor change in HBV mutant type in patients treated with anti-HBV drugs.

REFERENCES

1 Zoulim F. Detection of hepatitis B virus resistance to antivirals. *J Clin Virol* 2001;21:243–53
2 Papatheodoridis GV, Dimou E, Papadimitropoulos V. Nucleoside analogues for chronic hepatitis B: antiviral efficacy and viral resistance. *Am J Gastroenterol* 2002;97:1618–28
3 Gutfreund KS, Williams M, George R, *et al.* Genotypic succession of mutations of the hepatitis B virus polymerase associated with lamivudine resistance. *J Hepatol* 2000;33:469–75
4 Akuta N, Tsubota A, Suzuki F, *et al.* Long-term prognosis by lamivudine monotherapy for severe acute exacerbation in chronic hepatitis B infection: emergence of YMDD motif mutant and risk of breakthrough hepatitis – an open-cohort study. *J Hepatol* 2003;38:91–7
5 Maynard M, Parvaz P, Durantel S, *et al.* Sustained HBs seroconversion during lamivudine and adefovir dipivoxil combination therapy for lamivudine failure. *J Hepatol* 2005;42:279–81
6 Westland CE, Yang H, Delaney WE, *et al.* Activity of adefovir dipivoxil against all patterns of lamivudine-resistant hepatitis B viruses in patients. *J Viral Hepat* 2005;12:67–73
7 Perrillo R, Schiff E, Yoshida E, *et al.* Adefovir dipivoxil for the treatment of lamivudine-resistant hepatitis B mutants. *Hepatology* 2000;32:129–34
8 Barcena Marugan R, Cid Gomez L, Lopez Serrano P. Use of adefovir in the treatment of the chronic hepatitis B virus infection with resistance to lamivudine. *Transplant Proc* 2003;35:1841–3
9 Qaqish RB, Mattes KA, Ritchie DJ. Adefovir dipivoxil: a new antiviral agent for the treatment of hepatitis B virus infection. *Clin Ther* 2003;25:3084–99
10 Zoulim F. Mechanism of viral persistence and resistance to nucleoside and nucleotide analogs in chronic hepatitis B virus infection. *Antiviral Res* 2004;64:1–15
11 Allen MI, Deslauriers M, Andrews CW, *et al.* Lamivudine Clinical Investigation Group. Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. *Hepatology* 1998;27:1670–7
12 Fu L, Liu S-H, Cheng Y-C. Sensitivity of L-(-)2′,3′-dideoxythiacytidine resistant hepatitis B virus to other antiviral nucleoside analogues. *Biochem Pharmacol* 1999;57:1351–9
13 Kobayashi S, Shimada K, Suzuki H, Tanikawa K, Sata M. Development of a new method for detecting a mutation in the gene encoding hepatitis B virus reverse transcriptase active site (YMDD motif). *Hepatol Res* (the official journal of the Japan Society of Hepatology) 2000;17:31–42
14 Yuen M-F, Kato T, Mizokami M, *et al.* Clinical outcome and virologic profiles of severe hepatitis B exacerbation due to YMDD mutations. *J Hepatol* 2003;39:850–5

15 Yoshida S, Yamada Y, Fujisawa S, *et al.* Dilution-PCR method for wide-range quantitation of HBV DNA. *Acta Hepatologica Japonica* 2002;**43**:482–6
16 Natsuizaka M, Hige S, Ono Y, *et al.* Long-term follow-up of chronic hepatitis B after the emergence of mutations in the hepatitis B virus polymerase region. *J Viral Hepat* 2005;**12**:154–9
17 Jardi R, Buti M, Rodriguez-Frias F, *et al.* Rapid detection of lamivudine-resistant hepatitis B virus polymerase gene variants. *J Virol Methods* 1999;**83**:181–7
18 Stuyver L, Van Geyt C, De Gendt S, *et al.* Line probe assay for monitoring drug resistance in hepatitis B virus-infected patients during antiviral therapy. *J Clin Microbiol* 2000;**38**:702–7
19 Kirishima T, Okanoue T, Daimon Y, *et al.* Detection of YMDD mutant using a novel sensitive method in chronic liver disease type B patients before and during lamivudine treatment. *J Hepatol* 2002;**37**:259–65
20 Kutyavin IV, Afonina IA, Mills A, *et al.* 3'-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. *Nucleic Acids Res* 2000;**28**:655–61
21 Punia P, Cane P, Teo C-G, Saunders N. Quantitation of hepatitis B lamivudine resistant mutants by real-time amplification refractory mutation system PCR. *J Hepatol* 2004;**40**:986–92
22 Umeoka F, Iwasaki Y, Matsumura M, *et al.* Early detection and quantification of lamivudine-resistant hepatitis B virus mutants by fluorescent biprobe hybridization assay in lamivudine-treated patients. *J Gastroenterol* 2006;**41**:693–701
23 Ono-Nita SK, Kato N, Shiratori Y, *et al.* YMDD motif in hepatitis B virus DNA polymerase influences on replication and lamivudine resistance: a study by in vitro full-length viral DNA transfection. *Hepatology* 1999;**29**:939–45
24 Yadav V, Chu CK. Molecular mechanisms of adefovir sensitivity and resistance in HBV polymerase mutants: a molecular dynamics study. *Bioorg Med Chem Lett* 2004;**14**:4313–17

(Accepted 12 April 2007)

doi:10.1111/j.1440-1746.2008.05502.x

HEPATOLOGY

8-Hydroxy-2′-deoxy-guanosine is a risk factor for development of hepatocellular carcinoma in patients with chronic hepatitis C virus infection

Makoto Chuma, Shuhei Hige, Mitsuru Nakanishi, Koji Ogawa, Mitsuteru Natsuizaka, Yoichi Yamamoto and Masahiro Asaka

Department of Gastroentelology and Hematology, Hokkaido University Hospital, Sapporo, Japan

Key words
8-hydroxy-2′-deoxy-guanosine (8-OHdG), hepatitis C Virus, hepatocellular carcinoma.

Accepted for publication 27 February 2008.

Correspondence
Dr Makoto Chuma, Department of Gastroenterology and Hematology, Hokkaido University, Kita 15, Nishi 7, Kitaku, Sapporo 060-8638, Japan. Email: mchuuma@med.hokudai.ac.jp

Abstract

Background and Aim: Increased production of reactive oxygen species, which cause oxidative DNA damage, is considered to be related to hepatocarcinogenesis. 8-Hydroxy-2′-deoxy-guanosine (8-OHdG) is a useful marker of DNA damage induced by oxidative stress. The aim of this study was to determine whether expression of 8-OHdG is a risk factor for the development of hepatocellular carcinoma (HCC) in patients with hepatitis C virus (HCV) infection.

Methods: The expression of 8-OHdG in liver biopsy specimens was assessed immunohistochemically. In total, 104 patients with chronic HCV infection who were diagnosed on liver biopsy between January 1987 and December 2002 were studied retrospectively. Univariate and multivariate analyses using age, gender, habitual drinking, tobacco exposure, diabetes mellitus, serum alanine aminotransferase level, HCV genotype, hepatic fibrosis, inflammation, steatosis, and 8-OHdG expression in liver biopsy specimens were conducted to identify factors related to the development of HCC.

Results: On multivariate analysis, 8-OHdG and fibrosis were independent and significant risk factors for HCC development (relative risk, 2.48; $P = 0.023$; relative risk, 5.35; $P = 0.001$, respectively). Furthermore, the cumulative incidence rate of HCC in 39 patients with high 8-OHdG expression levels was significantly greater than that in 65 patients with low 8-OHdG expression levels ($P = 0.043$). In addition, liver 8-OHdG expression was correlated with hepatic inflammation.

Conclusions: 8-OHdG is a risk factor for the development of HCC in patients with chronic HCV infection. Patients with chronic HCV who express 8-OHdG should be monitored carefully for the development of HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide and one of the leading causes of cancer death in Japan.[1] During the past several decades, the incidence of HCC has increased substantially in Japan. The increase in the incidence of HCC has been attributed to the increased number of patients with hepatitis C virus (HCV)–associated liver cirrhosis.[2] Several risk factors for the development of HCV-associated HCC have been identified, including age,[3] gender,[4] total alcohol intake,[5,6] tobacco exposure,[6] diabetes mellitus,[7] cirrhosis,[5,8] irregular regeneration of hepatocytes,[9] HCV genotype,[10] elevated serum alanine aminotransferase (ALT),[11] and interferon (IFN) treatment.[12] Failure to achieve a sustained virological response (SVR) to IFN-based therapy, as well as preexisting advanced hepatic fibrosis and/or cirrhosis, are the major predictors for the development of HCC and mortality.[12] The identification of additional variables associated with the risk of developing HCC is important as this would help in designing preventive medical programs.

8-Hydroxy-2′-deoxy-guanosine (8-OHdG), a DNA base-modified product generated by reactive oxygen species, is a mutation prone to induce G-C to T-A transversion at DNA replication.[13] Previous studies have shown that 8-OHdG is a good marker of oxidative DNA damage,[14] and is implicated in carcinogenesis[14,15] and hepatocarcinogenesis.[16] 8-OHdG is also produced in chronic liver diseases, including chronic hepatitis C.[17] The objectives of this study were to investigate the relationship between 8-OHdG expression and histological findings (inflammation, fibrosis) in the liver and, using univariate and multivariate analyses, to determine whether 8-OHdG is an independent risk factor for the development of HCC in patients with chronic HCV infection.

Methods

Patients

Liver biopsies were obtained from 548 patients with no detectable HCC at Hokkaido University Hospital, Sapporo, Japan, from January 1987 to September 2002. Patients with bleeding tendencies or clinically evident cirrhosis associated with ascites or hepatic encephalopathy were excluded from the biopsy procedure. In this retrospective study, of the 548 patients who were assessed initially, 104 fulfilled the following criteria and were enrolled: (i) liver biopsy that showed histopathological features of chronic hepatitis or cirrhosis; (ii) HCV-RNA-positive based on the amplicor monitor assay[18] (Roche Diagnostic Systems, Tokyo, Japan); (iii) negative for hepatitis B surface antigen (HBsAg), antinuclear antibody (ANA), and antimitochondrial antibody (AMA) in the serum, based on radioimmunoassay and spot hybridization techniques; (iv) no sustained virological response to IFN therapy; (v) more than 60 months of follow-up at our hospital; and (vi) no occurrence of HCC within 6 months after liver biopsy. At the time of the liver biopsy, the physicians asked about alcohol drinking habits. Habitual heavy drinking was defined as an average daily consumption of an amount equivalent to 65 g of pure ethanol over a period of 5 years. Tobacco exposure was defined as ≥ 20 pack-years, because 1 pack (20 cigarettes)/day has been shown to be a risk for HCC over a 20-year period.[6] Diabetes mellitus was diagnosed on the basis of the American Diabetes Association guidelines.[19]

Serologic tests

Frozen-stored sera obtained from patients at the time of liver biopsy were tested. The serum HCV-RNA level was determined using an Amplicor GT HCV monitor (Roche Diagnostic Systems). HCV genotypes 1 and 2 were determined using a serologic genotyping assay.[20] In this assay, genotypes 1 and 2 correspond to genotype 1 (1a, 1b) and genotype 2 (2a, 2b) as proposed by Simmonds *et al.*[21]

Follow-up of patients

During follow-up, clinical evaluations and biochemical tests were performed every 1–3 months. Patients underwent liver ultrasonography or computed tomography at least every 6 months. HCC was diagnosed based on the American Association for the Study of Liver Disease guidelines.[22] The endpoint used in this study was the appearance of HCC; 30 September 2007 was used as the reference date. Ninety-five patients were followed until the endpoint was reached. The duration of follow-up was 61 to 204 months (mean observation time, 122 months). The patients' background characteristics at study entry are summarized in Table 1.

Histopathological examination of the liver

Liver histology was evaluated according to the degree of fibrosis and the degree of inflammatory activity. The degree of fibrosis (staging) and the degree of necroinflammatory activity (grading) were classified according to Desmet *et al.*[23] as follows: mild fibrosis included F0 (none), F1 (periportal expansion), and F2 (portoportal septa), and severe fibrosis included F3 (portocentral linkage or bridging fibrosis) and F4 (cirrhosis). Mild necroinflammatory activity included grade A1, while severe activity included grades A2 and A3. Hepatic steatosis was graded according to Brunt *et al.*[24] In short, steatosis observed in up to 33%, 33%–66%, and more than 66% of the liver histology was classified as grade 1, 2, and 3, respectively. If no hepatic steatosis was observed, grade 0 was assigned.

Table 1 Clinical and laboratory characteristics of 104 patients

Characteristic	No of patients
Gender (male/female)	44/60
Median age (years, mean ± SD)	50.5 ± 11.5
Habitual drinking (yes/no)	28/76
Tobacco exposure (yes/no)	18/86
Diabetes mellitus (yes/no)	19/85
ALT (IU/L, mean ± SD)	79.8 ± 50.9
HCV genotype (1/2/undetermined)	79/18/7
IFN (yes/no)	75/29

ALT, alanine aminotranferase; HCV, hepatitis C virus; IFN, interferon.

Immunohistochemistry

Immunohistochemical staining was done on formalin-fixed, paraffin-embedded tissue sections using an immunoperoxidase method, as described previously.[25] Briefly, each section was deparaffinized, rehydrated, and incubated with fresh 0.3% hydrogen peroxide in methanol for 30 min at room temperature and then washed in phosphate-buffered saline. Normal swine serum (Dako, Glostrup, Denmark) was applied for 30 min and then removed. The sections were incubated with mouse monoclonal antibodies for anti-8-OHdG antibody (Japan Institute for the Control of Aging, Shizuoka, Japan) at a dilution of 1 : 50 overnight at 4°C. The specimens were subsequently washed three times in phosphate-buffered saline, and then incubated with a secondary antibody for 30 min at room temperature. A Vectastatin Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) was used for antibody detection. The degree of 8-OHdG immunostaining was expressed as the labeling index (LI), based on the percentage of positive cells identified by dark brown staining of the nucleus per 1000 cells; the relationship between the LI and the development of HCC was examined.

Statistical analysis

The cumulative HCC incidence rates were calculated and plotted using the Kaplan–Meier method. Univariate and multivariate analyses of the risk ratios for the occurrence of HCC were done using Cox's proportional hazards regression analysis. The risk factors examined included age, gender, habitual heavy drinking, diabetes mellitus, mean follow-up serum ALT (≥ 90 IU/L or < 90 IU/L), staging (mild fibrosis or severe fibrosis), and grading (mild activity or severe activity). Differences between the two groups were analyzed using the log-rank test. All P values were two-tailed, and P-values < 0.05 were considered significant. Statistical analysis was performed using Stat View software (version 5.0; SAS Institute, Cary, NC, USA).

Journal of Gastroenterology and Hepatology **23** (2008) 1431–1436

Results

Histopathological findings

Examples of the histopathological findings are shown in Table 2. Based on the histopathological staging, five patients (4.8%) had F0 fibrosis, 45 patients (43.3%) had F1 fibrosis, 33 patients (31.7%) had F2 fibrosis, 15 patients (14.4%) had F3 fibrosis, and six patients (5.7%) had F4 fibrosis. Based on the histopathological grading, 45 patients (43.3%) had A1 necroinflammatory activity, 54 patients (51.9%) had A2 activity, and five patients (4.8%) had A3 activity. Hepatic steatosis was present in 25 (24%) of the 104 patients. Based on the histopathological grading, 79 patients (76%) had grade 0 steatosis, 22 patients (21.6%) had grade 1, 3 patients (2.9%) had grade 2, and no patients had grade 3 steatosis.

Hepatocyte expression of 8-OHdG

8-OHdG expression was detected in 62 (59.6%) of the 104 patients with chronic hepatitis C. However, 8-OHdG expression was very weak in 23 of the 62 cases; the 8-OHdG LI was less than 30% in these 23 cases. Thus, to objectively evaluate the relationship between quantitative expression of 8-OHdG and HCC development, 30% was defined as the 8-OHdG LI cut-off point. Nuclear expression of 8-OHdG in hepatocytes was especially abundant in periportal areas with piecemeal necrosis and prominent cell infiltration (Fig. 1).

Relationship between liver 8-OHdG LI and grading and staging

The 8-OHdG LI was correlated with the pathologic grading of necroinflammatory activity: the mean 8-OHdG LIs were 25.1% for A1, 34.8% for A2, and 48% for A3 (Fig. 2a). However, the 8-OHdG LI was not strongly correlated with pathologic staging of liver fibrosis: the mean 8-OHdG LI increased gradually as the stage increased from F0 (15%) to F1 (24.8%) and then F2 (39.1%), but the mean 8-OHdG LI values were similar in the F2 (39.1%), F3 (37.3%), and F4 (36.7%) stages (Fig. 2b).

Table 2 Histologic characteristics of 104 patients

Characteristic	No. of patients (%)
Distribution of stage of fibrosis (F)	
0	5 (4.8)
1	45 (43.3)
2	33 (31.7)
3	15 (14.4)
4	6 (5.7)
Distribution of grade of inflammation (A)	
1	45 (43.3)
2	54 (51.9)
3	5 (4.8)
Distribution of grade of steatosis	
0	79 (76.0)
1	122 (21.6)
2	23 (2.9)
3	30 (0)

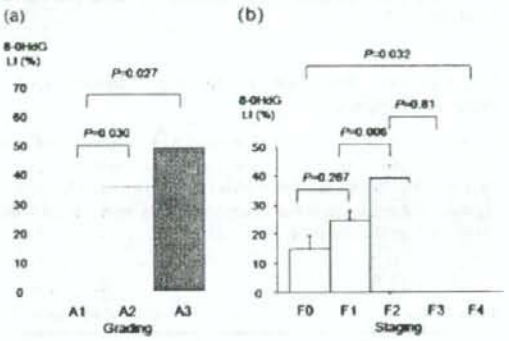

Figure 2 Differences in the 8-hydroxy-2'-deoxy-guanosine (8-OHdG) labeling index by liver biopsy grade (a) and stage (b).

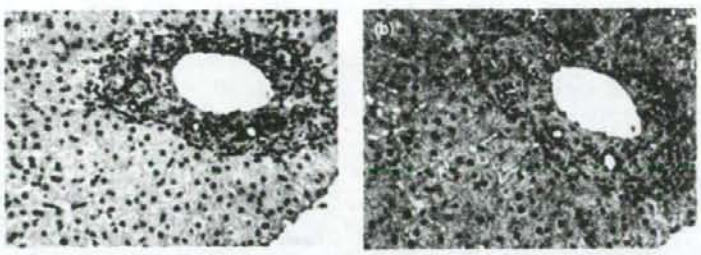

Figure 1 Immunohistochemical localization of 8-hydroxy-2'-deoxy-guanosine (8-OHdG) in patients with chronic hepatitis C (CH-C). Representative histology of CH-C (b) (hematoxylin–eosin stain, original magnification × 20), and 8-OHdG immunostaining of a serial section (a). Many 8-OHdG-positive hepatocytes are present in the portal area of tissue obtained from a patient with CH-C. Cells with a brown-stained nucleus were considered positive. In this patient, the positive rate was 70%.

Analysis of the clinicopathological factors affecting HCC development

During follow-up, 35 of the 104 patients (33.7%) developed HCC; 22 patients were diagnosed as having HCC based on the results of ultrasonography-guided biopsy, and 13 patients were diagnosed as having HCC based on the presence of the typical vascular pattern on dynamic imaging. The cumulative incidence rates of HCC were 14.7% at 7 years, 29.1% at 10 years, and 50.6% at 15 years after the liver biopsy. To determine the factors that may influence the development of HCC, a Cox's proportional hazards regression analysis was performed. On univariate analysis, the following three factors significantly affected the incidence of HCC: patient age at the time of liver biopsy (relative risk, 2.02; 95% confidence interval [CI], 1.03–3.97; $P = 0.042$), histopathological stage (fibrosis: relative risk, 4.10; 95% CI, 2.00–8.39; P = 0.001), and 8-OHdG expression (relative risk, 2.09; 95% CI, 1.07–4.11; $P = 0.031$) (Table 3). Gender, alcohol intake, tobacco exposure, diabetes mellitus, HCV genotype, serum ALT, and pathological grade were not correlated with HCC development. On multivariate analysis of the determinants of HCC using a Cox regression model, fibrosis (relative risk, 5.35; 95% CI, 1.96–14.6; $P = 0.001$) and 8-OHdG expression (relative risk, 2.48; 95% CI, 1.13–5.41; $P = 0.023$) were identified as statistically independent risk factors (Table 4).

Relationship between the liver 8-OHdG LI and HCC development

Overall, 39 (37.5%) of the 104 patients had a high 8-OHdG LI (30%). As shown in Figure 3, the cumulative incidence rate of HCC in these 39 patients with high 8-OHdG expression levels was significantly greater than that in the 65 patients with low 8-OHdG expression levels ($P = 0.043$).

Table 3 Univariate analysis of risk ratios for hepatocellular carcinoma

Characteristic	Risk ratio	95% CI	*P*-value
Gender (compared with female)	1.37	0.70–2.75	0.37
Age (compared with < 55)	2.02	1.03–3.97	0.042†
Habitual drinking (compared with no habitual drinking)	1.38	0.64–2.99	0.41
Tobacco exposure (compared with no tobacco exposure)	1.02	0.52–2.00	0.95
Diabetes mellitus (compared with absent)	1.30	0.61–3.00	0.49
HCV genotype (compared with genotype 2)	1.15	0.48–2.80	0.75
Mean ALT (compared with < 90)	1.23	0.62–2.44	0.56
Inflammation (compared with mild)	1.45	0.72–2.95	0.30
Fibrosis (compared with mild)	4.10	2.00–8.39	0.001†
Steatosis (compared with grade 0)	1.62	0.67–3.93	0.29
8-OHdG expression (compared with < 30%)	2.09	1.07–4.11	0.031†

†Statistically significant.
95% CI, 95% confidence interval; ALT, alanine aminotranferase; HCV, hepatitis C virus; IFN, interferon; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.

Discussion

The current study evaluated the incidence of HCC and analyzed the factors that affect the incidence of HCC in patients with HCV-related chronic liver disease diagnosed on liver biopsy. Patients who were sustained virological responders to IFN therapy were not enrolled because it is well known that the development of HCC in such patients is significantly suppressed.[11] On univariate and multivariate analyses, it was found that the 8-OHdG expression level and fibrosis were significant independent risk factors for

Table 4 Multivariate analysis of risk ratios for hepatocellular carcinoma

Characteristic	Risk ratio	95% CI	*P*-value
Gender (compared with female)	1.33	0.58–3.03	0.50
Age (compared with < 55)	2.00	0.88–4.44	0.10
Habitual drinking (compared with no habitual drinking)	1.48	0.60–3.65	0.40
Tobacco exposure (compared with no tobacco exposure)	1.17	0.31–4.40	0.82
Diabetes mellitus (compared with absent)	1.30	0.35–4.40	0.73
HCV genotype (compared with genotype 2)	0.92	0.29–3.00	0.89
Mean ALT (compared with < 90)	1.13	0.30–4.29	0.86
Inflammation (compared with mild)	1.51	0.67–3.36	0.31
Fibrosis (compared with mild)	5.35	1.96–14.6	0.001†
Steatosis (compared with grade 0)	1.50	0.66–3.40	0.33
8-OHdG expression (compared with < 30%)	2.48	1.13–5.41	0.023†

†Statistically significant.
95% CI, 95% confidence interval; ALT, alanine aminotranferase; HCV, hepatitis C virus; IFN, interferon; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.

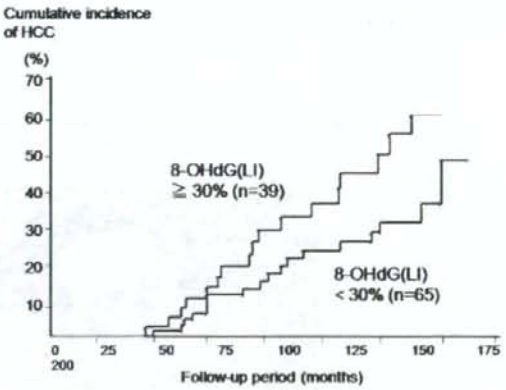

Figure 3 Cumulative incidence rates of hepatocellular carcinoma (HCC) based on the prevalence of 8-hydroxy-2′-deoxy-guanosine (8-OHdG) expression in patients with chronic hepatitis C. The cumulative incidence rate of HCC in 39 patients with high 8-OHdG expression levels was significantly higher than that in 65 patients with low 8-OHdG expression levels ($P = 0.043$).

Journal of Gastroenterology and Hepatology **23** (2008) 1431–1436 © 2008 The Authors
Journal compilation © 2008 Journal of Gastroenterology and Hepatology Foundation and Blackwell Publishing Asia Pty Ltd

HCC development. Age, gender, total alcohol intake, tobacco exposure, diabetes mellitus, HCV genotype, and elevated serum ALT were not correlated with HCC development. A relationship between the 8-OHdG expression level and HCC development was also demonstrated.

It is not clear why the rate of HCC occurrence was high in patients with high liver 8-OHdG expression. 8-OHdG is one of the main markers of oxidative DNA injury. During hepatocarcinogenesis, the increased production of reactive oxygen species is thought to cause oxidative DNA damage. Studies of hepatocarcinogenesis related to the presence of chemical carcinogens, such as peroxisome proliferators and N-nitrosodiethylamine, have also shown that 8-OHdG is induced during liver DNA damage.[16] 8-OHdG is considered to be a useful indicator for investigating the involvement of active oxygen in hepatocarcinogenesis.[26] The expression of 8-OHdG has been reported in the liver of patients with chronic hepatitis C,[27,28] cirrhosis,[29] and HCC.[30] These reports noted that there is a relationship between the 8-OHdG expression level in the noncancerous region and postoperative recurrence of HCC in the remnant liver.[31] It was also found that the number of 8-OHdG-positive hepatocytes was greater in HCC than in chronic hepatitis.[30] Based on our results and these reports, it is possible that 8-OHdG-positive hepatocytes have a high-grade malignancy potential in HCC and that 8-OHdG could thus be a useful marker of HCC development. In the present study, the 8-OHdG LI was shown to be correlated with the pathologic inflammation, but the univariate and multivariate analyses showed that pathologic inflammation did not affect the incidence of HCC. The reason for this discrepancy is not clear. In several reports in which it was reported that 8-OHdG caused by oxidative DNA damage was involved in hepatocarcinogenesis, Sekoguchi *et al.* showed that 8-OHdG-positive hepatocytes had shorter telomeres than 8-OHdG-negative hepatocytes,[32] Kinoshita *et al.* reported formation of 8-OHdG and cell-cycle arrest in the rat liver via generation of oxidative stress in association with p21 and cyclin D1,[33] and Shen *et al.* reported an association between 8-OHdG and cell proliferation.[34] On the basis of these reports, 8-OHdG formation may be involved in alteration of cellular proliferation and apoptosis in the liver; these phenomena do not always accompany necroinflammation of the liver. Thus, the presence of 8-OHdG is considered to not only reflect the severity of liver cell injury (inflammation, fibrosis), but also to induce gene mutations, which implies that the presence of 8-OHdG is closely involved with hepatocarcinogenesis. Therefore, it is likely that 8-OHdG plays a role in hepatocarcinogenesis via a mechanism other than inflammation. Further analyses are required to identify the role of 8-OHdG in hepatocarcinogenesis.

In this study, the relationship between serum and liver tissue levels of 8-OHdG was investigated. Although there was a tendency for serum and tissue 8-OHdG levels to be correlated, it was not significant (data not shown).

The results of the present study indicate that the presence of 8-OHdG expression at the time of liver biopsy could be a useful marker of the development of HCC. Thus, these results emphasize the need for careful monitoring for the development of HCC in patients with chronic HCV and 8-OHdG expression. Given the small number of patients included in the present study, the current findings need to be confirmed in a larger population of patients with chronic HCV infection.

References

1 Kiyosawa K, Umemura T, Ichijo T *et al.* Hepatocellular carcinoma: recent trends in Japan. *Gastroenterology* 2004; **127**: S17–26.
2 Lai MM. Hepatitis C virus proteins: direct link to hepatic oxidative stress, steatosis, carcinogenesis and more. *Gastroenterology* 2002; **122**: 568–71.
3 Kasahara A, Hayashi N, Mochizuki K *et al.* Risk factors for hepatocellular carcinoma and its incidence after interferon treatment in patients with chronic hepatitis C. *Hepatology* 1998; **27**: 1394–402.
4 Makiyama A, Itoh Y, Kasahara A *et al.* Characteristics of patients with chronic hepatitis C who develop hepatocellular carcinoma after a sustained response to interferon therapy. *Cancer* 2004; **101**: 1616–22.
5 Tokita H, Fukui H, Tanaka A *et al.* Risk factors for the development of hepatocellular carcinoma among patients with chronic hepatitis C who achieved a sustained virological response to interferon therapy. *J. Gastroenterol. Hepatol.* 2005; **20**: 752–8.
6 Marrero JA, Fontana RJ, Fu S, Conjeevaram HS, Su GL, Lok AS. Alcohol, tobacco and obesity are synergistic risk factors for hepatocellular carcinoma. *J. Hepatol.* 2005; **42**: 218–24.
7 El-Serag HB, Richardson PA, Everhart JE. The role of diabetes in hepatocellular carcinoma: a case-control study among United States Veterans. *Am. J. Gastroenterol.* 2001; **96**: 2462–7.
8 Fattovich G, Stroffolini T, Zagni I *et al.* Hepatocellular carcinoma in cirrhosis: incidence and risk factors. *Gastroenterology* 2004; **127**: S35–50.
9 Shibata M, Morizane T, Uchida T *et al.* Irregular regenera-tion of hepatocytes and risk of hepatocellular carcinoma in chronic hepatitis and cirrhosis with hepatitis-C-virus infec-tion. *Lancet* 1998; **351**: 1773–7.
10 Bruno S, Stroffolini T, Colombo M *et al.* Sustained virological response to interferon-alpha is associated with improved outcome in HCV-related cirrhosis: a retrospective study. *Hepatology* 2007; **45**: 579–87.
11 Tarao K, Rino Y, Ohkawa S *et al.* Close association between high serum alanine aminotransferase levels and multicentric hepatocarcinogenesis in patients with hepatitis C virus-associated cirrhosis. *Cancer* 2002; **94**: 1787–95.
12 Yoshida H, Arakawa Y, Sata M *et al.* Interferon therapy prolonged life expectancy among chronic hepatitis C patients. *Gastroenterology* 2002; **123**: 483–91.
13 Shibutani S, Takeshita M, Grollman AP. Insertion of specific basesduring DNA synthesis past the oxidation-damaged base 8-oxdG. *Nature* 1991; **349**: 431–4.
14 Kasai H. Analysis of a form of oxidative DNA damage 8-hydroxy-2-deoxyguanosine, as a marker of cellular oxidative stress during carci-nogenesis. *Mutat. Res.* 1997; **387**: 147–63.
15 Chen X, Ding YW, Yang G, Bondoc F, Lee MJ, Yang CS. Oxidative damage in an esophageal adenocarcinoma model with rats. *Carcinogenesis* 2000; **21**: 257–63.
16 Nakae D, Kobayashi Y, Akai H *et al.* Involvement of 8-hydroxyguanine formation in the initiation of rat liver carcinogenesis by low dose levels of N-nitrosodiethylamine. *Cancer Res.* 1997; **57**: 1281–7.
17 Kitada T, Seki S, Iwai S, Yamada T, Sakaguti H, Wakasa K. In situ detection of oxidative DNA damage, 8-hydroxydeoxyguanosine, in chronic human liver disease. *J. Hepatol.* 2001; **35**: 613–8.
18 Albadalejo J, Alonso R, Antinozzi R *et al.* Multicenter evaluation of the COBAS AMPLICOR HCV assay, an integrated PCR system for rapid detection of hepatitis C virus RNA in the diagnostic laboratory. *J. Clin. Microbiol.* 1998; **36**: 862–5.
19 Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* 1997; **20**: 1183–97.

20 Tsukiyama-Kohara K, Yamaguchi K, Maki N *et al.* Antigenicities of group 1 and 2 hepatitis C virus polypeptides: molecular basis of diagnosis. *Virology* 1993; **192**: 430–7.
21 Simmonds P, Alberti A, Alter HJ *et al.* A proposed system for the nomenclature of hepatitis C virus genotypes. *Hepatology* 1994; **19**: 1321–4.
22 Bruix J, Sherman M; Practice Guidelines Committee, American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma. *Hepatology* 2005; **42**: 1208–36.
23 Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am. J. Gastroenterol.* 1999; **94**: 2467–74.
24 Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994; **19**: 1513–20.
25 Altini M, Coleman H, Doglioni C, Favia G, Maiorano E. Calretinin expression in ameloblastomas. *Histopathology* 2000; **37**: 27–32.
26 Schwarz KB, Kew M, Klein A *et al.* Increased hepatic oxidative DNA damage in patients with hepatocellular carcinoma. *Dig. Dis. Sci.* 2001; **46**: 2173–8.
27 Horiike S, Kawanishi S, Kaito M *et al.* Accumulation of 8-nitroguanine in the liver of patients with chronic hepatitis C. *J. Hepatol.* 2005; **43**: 403–10.
28 Cardin R, Saccoccio G, Masutti F, Bellentani S, Farinati F, Tiribelli C. DNA oxidative damage in leukocytes correlates with the severity of HCV-related liver disease: validation in an open population study. *J. Hepatol.* 2001; **34**: 587–92.
29 Marotta F, Yoshida C, Barreto R, Naito Y, Packer L. Oxidative-inflammatory damage in cirrhosis: effect of vitamin E and a fermented papaya preparation. *J. Gastroenterol. Hepatol.* 2007; **22**: 697–703.
30 Ichiba M, Maeta Y, Mukoyama T *et al.* Expression of 8-hydroxy-2′-deoxyguanosine in chronic liver disease and hepatocellular carcinoma. *Liver Int.* 2003; **23**: 338–45.
31 Matsumoto K, Satoh Y, Sugo H *et al.* Immunohistochemical study of the relationship between 8-hydroxy-2′-deoxyguanosine levels in noncancerous region and postoperative recurrence of hepatocellular carcinoma in remnant liver. *Hepatol. Res.* 2003; **25**: 435–41.
32 Sekoguchi S, Nakajima T, Moriguchi M *et al.* Role of cell-cycle turnover and oxidative stress in telomere shortening and cellular senescence in patients with chronic hepatitis C. *J. Gastroenterol. Hepatol.* 2007; **22**: 182–90.
33 Kinoshita A, Wanibuchi H, Imaoka S *et al.* Formation of 8-hydroxydeoxyguanosine and cell-cycle arrest in the rat liver via generation of oxidative stress by phenobarbital: association with expression profiles of p21(WAF1/Cip1), cyclin D1 and Ogg1. *Carcinogenesis* 2002; **23**: 341–9.
34 Shen J, Wanibuchi H, Salim EI *et al.* Liver tumorigenicity of trimethylarsine oxide in male Fischer 344 rats—association with oxidative DNA damage and enhanced cell proliferation. *Carcinogenesis* 2003; **24**: 1827–35.

臨牀消化器内科 2008 Vol.23 No.1
©日本メディカルセンター

検査値の読み方 連載

血液中で測定されるHCV-RNA定量値の意味

髭 修平* 中馬 誠* 山本 桂子*
山本 洋一* 中西 満* 浅香 正博*

はじめに

C型肝炎患者の臨床的検討を行う際にC型肝炎ウイルス(HCV)関連マーカーの測定は重要である．なかでも，血液中のウイルス核酸(HCV-RNA)量の測定は，抗ウイルス治療の効果予測，治療開始後の反応性の評価，などに大きな意義を有する．しかし，HCVは肝臓内で増幅し，その一部が血液中に放出されていることを考慮すると，通常，われわれが測定しているHCV-RNA量は，体内・肝内のHCVに関する間接情報である．さらに，HCVの体内動態は非常にダイナミックであることも明らかになってきた．本稿では，臨床検査として測定可能な「HCV-RNA量」値の意味について，われわれのデータを示して考察する．

Ⅰ．HCV-RNA量の変動

C型肝炎患者の経過中のHCV-RNA量の経時的変化について，当科における検査結果を示す．C型慢性肝疾患患者のなかで，1年以上，月1回のHCV-RNA量定量測定を行った138例についてHCV-RNA量の測定結果を示す．

対象症例は慢性肝炎121例，肝硬変症17例，平均年齢は62.8歳，男女比は60：78であった．HCVグループ別には，1型107例，2型27例，判定不能4例であった．肝庇護療法(強力ネオミノファーゲンCあるいはウルソデオキシコール酸)施行例では，観察開始時から内容に変更のない例を選択した．

HCV-RNA量は，基本的には分岐プローブ法(b-DNA法)で測定し，0.24Meq/m*l*の測定感度未満となる例ではPCR法(Amplicor法)で測定した．その結果，b-DNA測定可能例は111例(1型98例，2型9例，判定不能4例)，Amplicor法測定例は27例(1型19例，2型8例)で，b-DNA法では測定感度以下になる比較的低値例は2型に有意に高率であった．測定したHCV-RNA量実測値は対数変換し，観察期間中のHCV-RNA量の変動を，平均値(M)，標準偏差(SD)，最高値と最低値の差(D)により評価した．

Key words: HCV-RNA量，HCV体内動態，肝組織中HCV，FSSA法，DFPP

Shuhei Hige／Makoto Chuma／Keiko Yamamoto／Yoichi Yamamoto／Mitsuru Nakanishi／Masahiro Asaka
*北海道大学大学院医学研究科消化器内科学(〒060-8638 北海道札幌市北区北15条西7丁目)

0911-601X/08/JCLS

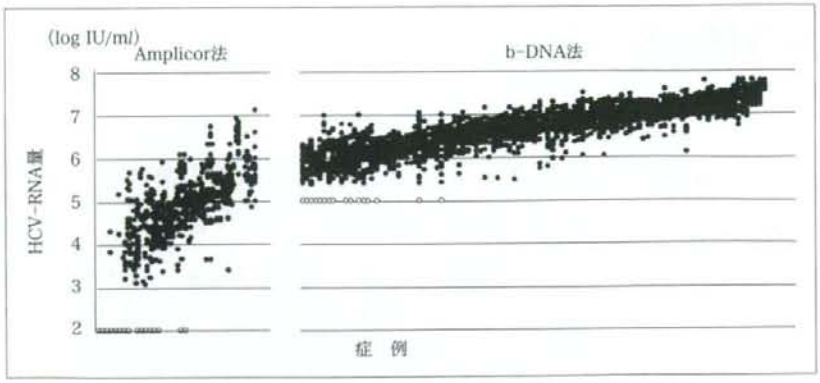

図1 C型慢性肝疾患患者のHCV-RNA量の変動
観察期間中の平均HCV-RNA値の低い順に症例を横軸に並べ，各症例の期間中の実測値を縦軸にプロットした(○は測定感度以下)．

実際のHCV-RNA量測定結果を**図1**に示す．1～2年の観察期間内で，HCV-RNA量の変動は数倍以内の例が多く，5～10倍以上の比較的大きな変動を示した例は少数であった．HCV-RNA量の平均値(M)分布では，b-DNA量測定例では6(log IU/ml)台が52例(51.0%)，Amplicor測定例では4(log IU/ml)台が11例(44.0%)と最多であった．標準偏差(SD)の分布では，0.1が48例(41.7%)，0.2が29例(25.2%)，0.3が13例(11.3%)で，SD値0.3以下の症例が約80%を占めた．一方，SD値が0.7以上を示す変動の多い症例は11例で全体の1割程度であった．M値とSD値に有意な逆相関($R^2=0.67$, $p<0.05$)を認め，HCV-RNA量が低値な例ほど経時的変動が大きい傾向を示した．D値(観察期間中の最大値と最小値の差)が1.0(log IU/ml)以上を示した例も，b-DNA法27例(29.0%)，Amplicor法18例(81.8%)と，後者のウイルス量低値例で変動が大きいことを示した．

この結果は，治療内容を含めて変化のない，多くのC型肝炎症例では，経過中2～3倍以上のHCV-RNA量変動はよく観察されることを示している．副腎皮質ステロイドや免疫抑制剤などがHCV-RNA量に影響するか議論になることもあるが，その場合も，本稿に示されるようなC型肝炎患者のHCV-RNA量のベースラインの変動状態を理解したうえで評価することが望まれる．また，これらの症例の多くはウイルス量の変動と血清ALT値の変化に関連が認められず，経過中の評価は別個に行う必要があることにも留意すべきである．

Amplicor法で測定したHCV-RNA量低値例では経過中の変動幅が比較的大きい傾向を示したが，このような症例にはウイルス量低値の時期があることを示しており，抗ウイルス治療開始のタイミングをはかる際の参考になる．HCV-RNA量低値群にはHCV-RNA量とALT値の変動が連動している症例があり，ウイルス量が低値でRNA量とALT値の連動を認めた例は11例(8.0%)であった．このような例でALT値からRNAレベルが推測可能な場合がある．

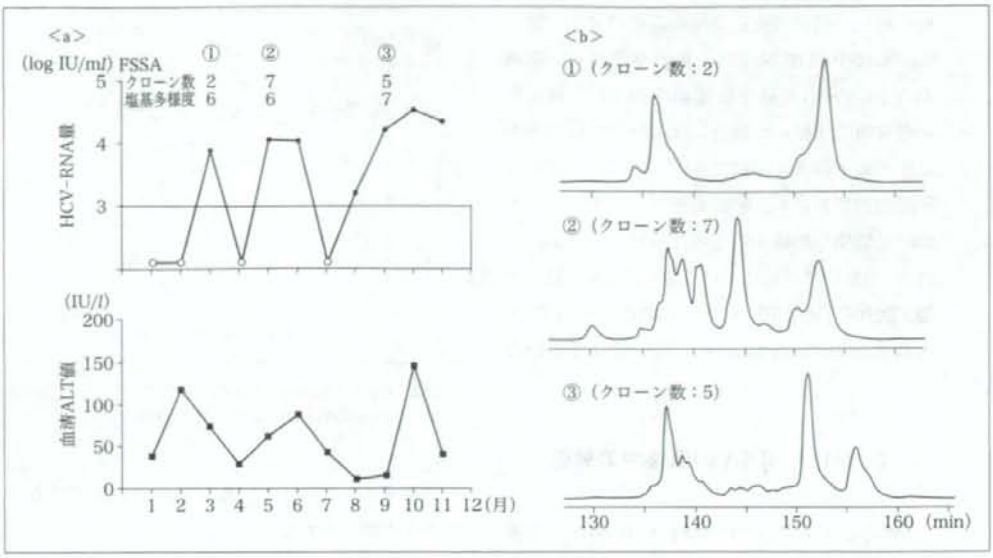

図2 ウイルス量の変動幅の大きい症例
a：HCV-RNA 量と ALT 値変動の関連：①～③は，FSSA 法による HCV 変異の測定点を示す．
b：SSCP 法による電気移動度の違いによる HCV クローン数の測定．

Ⅱ．HCV-RNA 量の変動と quasispecies の変化

ウイルス量変動の大きい症例の，HCV 超可変領域(hypervariable region；HVR-1)における経時的変化を FSSA 法〔fluorescence single strand conformation polymorphism(SSCP)and sequence analysis〕を用いて検討した．本法は，SSCP 法により 1 本鎖 DNA 断片を電気泳動の移動度の違いにより分離して測定領域から検出されたクローン数を算出し，さらに direct sequence 法で測定領域中の変異率を塩基多様度として示すことで，HCV 変異の状態を解析するものである．HCV-RNA/ALT 連動例について，肝炎再燃時ごとの HCV を FSSA 法で解析した結果，再燃時ごとに HCV population は変化をしていることがウイルス学的に示された．

1 例を**図 2** に示す．

【症　例】

26 歳，女性．グループ 2 型の C 型慢性肝炎例である．経過中に HCV-RNA 量は 10 倍以上の変動を繰り返し，ALT もウイルス量に連動していた．それぞれのウイルス量増加時の血清から FSSA 法による HCV 変異の解析をしたところ，各肝炎再燃時のウイルスの population は異なっていた．したがって，このような例の HCV-RNA 量変動は，単にウイルス量の増減ではなく，quasispecies の変化と関連する経時的変化である可能性がある．

Ⅲ．血液中と組織内の HCV-RNA

HCV は体内では，おもに肝細胞内で増幅して血液中に放出されることが知られている．わ

れわれが，通常の検査で知ることのできる情報は，流血中の HCV-RNA 量のみである．血液中の HCV-RNA 量と肝組織内の RNA 量を同一容積内で比較した場合には，肝内には血液中に比べ数十倍多く存在する[1]．さらに，HCV は肝細胞内以外にも，末梢血単核球(PBMC)に能動的に感染し増幅すると考えられているが，体内の PBMC プールにおける HCV-RNA 量の評価，血液中 HCV-RNA 量との関連など，PBMC を含めた HCV dynamics に関しては報告がない．

Ⅳ．HCV-RNA の血液中の動態

Neumann ら[2]は，interferon(IFN)α2b を投与した C 型慢性肝炎患者の HCV dynamics を mathematical model を用いて解析した．HCV-RNA 量を V とし，血液中 HCV-RNA 量は肝細胞内で増幅し放出されるウイルス量と血液中から排除されるウイルス量の差で規定される．

すなわち経時的変化(dV/dt)は，

$dV/dt = pI - cV$ と示される．

上式においては，p：1 日当りの感染肝細胞内 HCV 産生率，I：感染肝細胞数，c：ウイルス消失率(定数)である．

また，β：感染肝細胞への感染率(定数)，δ：感染肝細胞の消失率(定数)，T：肝細胞数，s：肝細胞増殖率(定数)，d：肝細胞消失率(定数)と表した場合，

$dT/dt = s - dT - (1-\eta)\beta VT$

$dI/dt = (1-\eta)\beta VT - \delta I$

$dV/dt = (1-\varepsilon)pI - cV$

と示される．なお，上式の$(1-\varepsilon)$はウイルス粒子産生率を示し，$(1-\eta)$は HCV の *de novo* 感染率を表している．

Neumann らは，上記の解析を IFNα2b 投与後の慢性肝炎患者で行い，IFN のおもな抗ウイルス効果が，ウイルスの複製や放出の抑制であることを明らかにした．

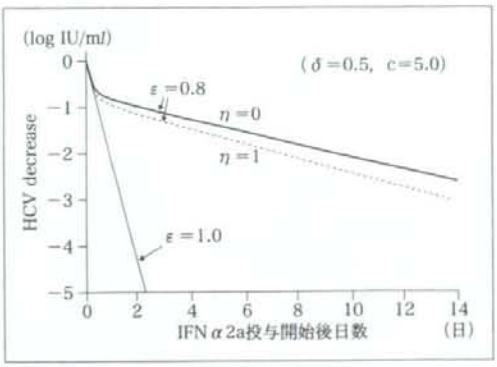

図 3 mathematical model から推定される IFNα2b 投与開始後の HCV-RNA の減少

上式のパラメータを変えた場合の IFNα2b 投与後 HCV 減少パターンにつき**図 3** に示す．ウイルス複製がまったく行われないと仮定した場合($\varepsilon=1$)，IFN 投与後に HCV-RNA は直線的に減少する．しかし，ウイルス複製が行われている体内の状態($\varepsilon \neq 1$)では，HCV-RNA 量の減少は図 3 のような二相性を示す．さらに，HCV の再感染の有無(η：0〜1)も HCV-RNA 減少に影響することが示されている．

血液中の HCV-RNA 量は，肝細胞内でのウイルスの産生と細胞外への放出，血液中でのウイルスの消失，肝細胞へのウイルスの再感染，感染肝細胞の破壊・排除などによるウイルス動態のバランスの結果として規定されるものと考えられる．Neumann らの解析では，HCV の産生および消失は 1 日に 10^{12}個程度と算出され，無治療状態では体内のダイナミックな動きが示された．一方で，産生・消失のバランスは動的には比較的安定し，総体としては定常状態を示しているものと考えられる．

V. 二重濾過血漿交換(DFPP)における HCV-RNA 量の解析

DFPP とは，患者血液を一次膜で血球と血漿に分離し，二次膜で血漿からウイルスなど不要な物質を除去する血漿浄化法である．わが国では，多発性骨髄腫，原発性マクログロブリン血症などに対し保険適応を有し，臨床応用されている．DFPP の二次膜の孔は最大 40 nm であり，径が 55～65 nm である HCV 粒子は二次膜の孔を通過できずに排除されると考えられる．Yamashita ら[3]は，C 型慢性肝炎患者の IFNα2b＋リバビリン治療に DFPP を併用し，安全性と治療初期の有効性を報告し，Fujiwara ら[4]は C 型慢性肝炎に対する IFNα2b 治療に DFPP を併用し，治療初期の HCV-RNA 量減量効果や substained viral response (SVR) において有用であることを報告した．

DFPP は HCV を物理的に排除する治療法で，二次膜通過後の濾液中 HCV-RNA は，ほとんどの例で測定感度以下となる．本法は肝細胞における HCV の複製や放出を抑制する IFN とは作用機序が異なり，前述した HCV の体内動態を解析する際に，新たな視点を導入できる可能性がある．多くの症例では DFPP 単独治療前後に HCV-RNA 量は低下するが，一部に開始前よりも上昇する症例が認められた．この違いは，ウイルスの増殖力の差や，肝臓と循環血液中の間の turn over の差などによる可能性が考えられ，DFPP が個々の症例における HCV 体内動態の解析手段になりうることが示された．

ま と め

HCV はおもに肝細胞内で増幅され血液中に放出されている．HCV は体内ではダイナミックな動態を示しているが，血液中 HCV-RNA 量はそのなかで定常状態を維持していることが示された．血液中 HCV-RNA 量は，このような体内動態の結果が示されていることを理解したうえで評価すべきである．

文　献

1) 髭　修平：血清および肝組織内 C 型肝炎ウイルス遺伝子検出の臨床的意義．北海道医誌 69；1382-1398，1994
2) Neumann, A. U., Nancy, P. L., Dahari, H., et al.: Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-α therapy. Science 282；103-107, 1998
3) Yamashita, T., Arai, K., Sakai, A., et al.: Virological effects and safety of combined double filtration plasmapheresis (DFPP) and interferon therapy in patients with chronic hepatitis C：a preliminary study. Hepatol. Res. 36；167-175, 2006
4) Fujiwara, K., Kaneko, S., Kakumu, S., et al.: Double filtration plasmapheresis and interferon combination therapy for chronic hepatitis C patients with genotype 1 and high viral load. Hepatol. Res. 37；701-710, 2007

特集 肝の線維化を探る

線維化の病態

ウイルス肝炎（壊死炎症の繰り返し）の線維進展

髭　　修平*

索引用語：肝線維化，ウイルス肝炎，壊死炎症反応，アポトーシス，staging

1 はじめに

肝臓は，肝細胞および胆管細胞の上皮組織と支持組織からなりたっており，本来の構成要素として種々の程度に線維結合織が含まれている．結合組織は，肝臓の支持組織のひとつであるが，基質(matrix)とその中に埋まっている細胞および線維からなる．この線維には，膠原線維，細網線維，弾性線維がある．

"肝線維化"とは，肝内で線維が異常に増加した状態であるが，ウイルス性肝炎の病因や病態の解明が進み，線維化の機序や評価も明らかにされてきた．本稿では臨床的な意義を含めて概説する．

2 線維化の病態

肝の線維化は，その形成機序により病態を分けてみることができる．まず，刺激に反応した線維増生の有無からみて，受動的線維化と能動的線維化とがある．前者では，脱落した肝細胞の枠としてあった線維が集合し，結果的に線維化が認められるようになる．後者では，線維産生細胞が積極的にプロコラーゲンを産生し細胞外に線維が沈着するものである．実際には両者が複合して線維化が生じていることが多い．

また，先行する組織障害が明らかでないまま，膠原線維産生細胞が直接刺激を受けて積極的に線維が増殖する1次的線維化と，先行する組織障害に対して反応性にコラーゲンが増殖する2次的線維化とに分類することもできる．

3 ウイルス性肝炎における肝の線維化

ウイルス性肝炎に伴う肝臓の線維化は，肝内の慢性的な炎症による組織障害に伴って2次的に起こる．肝障害が一過性の場合，肝細胞の再生が十分であれば，細胞外マトリックスは吸収されて障害組織は痕を残さない．しかし，慢性的に壊死炎症反応が繰り返されるウイルス性肝炎においては，肝再生よりも結合織の増生が勝り，その結果，細胞外マトリックスは吸収されずに沈着し，線維化が進

Syuhei HIGE: Progression of liver fibrosis in viral hepatitis by repeated necro-inflammation
*北海道大学消化器内科学［〒060-8638 札幌市北区北15条西7丁目］

行していくことになる[1]．そして，コラーゲン沈着量の増加は肝臓の組織構築を改変させていく．

ウイルス性肝炎おける肝細胞死は，壊死(necrosis)だけではなく，アポトーシスも大きく関与していることが明らかとなってきた．肝細胞のアポトーシスから肝線維化への進展に関しては，以下の機序が考えられている[2]．アポトーシスを起こし分解された肝細胞からはアポトーシス小体が形成されるが，この小体を取り込んだ周囲のクッパー細胞や肝星細胞から，強力な線維化促進因子であるTGF-βやFas ligandなどの分泌が亢進する．これらの細胞が持続的に活性化されると，IL-8やMIP-2（macrophage inflammatory protein-2）などのCXCケモカインが誘導され，肝炎は増強しアポトーシスがさらに進行する．このような"feed-forward-loop"が形成された状態で，肝星細胞はさらに活性化を受ける．活性化した星細胞は筋線維芽細胞に形質転換するが，この細胞からTGF-βの他にも，抗アポトーシス作用を持つTIMP-1（tissue inhibitor of metalloproteinase-1）などが分泌される．その結果，活性化星細胞の分解が抑制され，細胞外マトリックスの蓄積が進行する．

4 B型肝炎とC型肝炎―組織像の違い

肝の線維化は持続する炎症の結果であり，ウイルス性慢性肝炎の組織所見は基本的には同じである．しかし，起因ウイルスによる経過・病態の違いを反映し，B型肝炎とC型肝炎の組織像にも違いが認められる．

B型肝炎では，C型肝炎に比べて活動期に壊死炎症反応が比較的強く，線維化が目立たない段階からinterface hepatitisの所見を認める頻度も高い．また，肝細胞の再生傾向が強く，結節形成も目立つ．広範な壊死炎症反応のために短期間で肝硬変まで進展する例も観察される．C型肝炎では，壊死炎症反応は比較的弱く，再生活動・結節形成傾向も乏しい．長期間にわたり炎症が持続することで徐々に病期が進展し，肝硬変への進展過程で膠原線維の沈着がより強くなる[3]．また，Disse腔や類洞に線維化の目立つ症例もあり，アルコール性肝障害に類似することもある．

同様に，線維化，再生結節形成が進んだ肝硬変においても，起因ウイルスによる差を認める．B型肝硬変においては，比較的大きめの再生結節を認め，線維性隔壁は狭い例が多い．一方，C型肝硬変では小さな結節を呈するものが多く，さらに一旦形成された再生結節をさらに細分割するような線維化がみられる．C型では門脈域および門脈域周囲の炎症細胞浸潤が比較的強く，肝硬変の進行に伴い線維化はさらに強くなる．その結果，線維性隔壁は不規則に厚くなる傾向を認める[4]．

5 慢性肝炎の組織診断基準の変遷と線維化の評価

慢性肝炎における肝臓の線維化が，より客観的に評価可能となったのは，肝組織診断基準の改訂によるところが大きい．従来は記述的であった肝組織の病理学的所見がスコア化された結果，肝の炎症や線維化の程度をより客観的に表現することが可能となった．

慢性肝炎の組織分類について，1990年頃までは1970年前後に提唱された分類(わが国では犬山分類，欧米ではヨーロッパ分類)が基本として利用されてきた．1981年にはKnodellら[5]により，肝組織病変の程度をスコア化して表現するHAI（histological activity index）スコアの手法がはじめて提唱され，