binding site [37], and is indispensable for AFP promoter function. However, it is possible that the degree of AFP promoter activity is also affected by expression levels of several transcription factors, in addition to HNF-1 and NF-I, their post-translational modifications (including phosphorylation), and interactions.

The AFP gene is frequently re-expressed in hepatocellular carcinomas (HCC), and serum AFP assays have led to the detection of HCCs at an early stage [6]. This indicates that the AFP regulatory sequences have the potential to express the cytostatic or cytotoxic genes in a hepatoma-specific manner. We previously reported on hepatoma-specific gene therapy using the human AFP 0.3-kb promoter alone to target AFP-producing hepatoma cells [24,38,39]. It has become possible, however, to clinically detect HCCs at an early stage, and AFP expression in these tumors has been found to be relatively low [6]. Additionally, without the far upstream enhancer regions, the activity of the AFP promoter alone is not strong enough to induce an anti-tumor effect on low- or non-AFP producing hepatoma cells [24,39]. Therefore, enhancement of the AFP promoter activity is likely required to induce a therapeutic effect. Recently, we have proposed two strategies to increase expression of therapeutic genes driven from the AFP promoter: the variant-type human AFP promoter having a −119g → a HNF-1 binding site mutation, and the hypoxia-inducible enhancer linked to the wild-type AFP promoter [25,40]. Both approaches successfully induced an increase in anti-tumor effects in a hepatoma-specific manner. In the present study, however, the variant-type AFP promoter was also active in adult mouse liver, indicating that gene therapy using the variant-type AFP promoter has the potential to induce cytotoxic effects not only in hepatoma cells, but also in normal hepatocytes. Therefore, when using the variant-type AFP promoter, hepatoma-specific gene transfer or therapeutic genes that function specifically in malignant cells should be used. Alternatively, a combination of wild-type AFP promoter and tumor-specific enhancers, which are activated by hypoxia or hypoglycemia, may be more suitable for gene therapy targeting for HCCs producing varied amounts of AFP [40].

In conclusion, we report two unrelated Japanese HPAFP families with a heterozygous −119g → a substitution in the distal HNF-1 binding site of the AFP promoter. Further elucidation of the molecular mechanisms of HPAFP should increase our understanding of the developmental regulation of AFP transcription, as well as aid in developing strategies for HCC-targeting gene therapy.

## Acknowledgments

We thank Ms. Yuka Takahama and Ms. Kana Ohara for their technical assistance. This work was supported in part by Grants-in-Aid from the Ministry of Science, Education, Sports, and Culture, and the Ministry of Health, Labor, and Welfare of Japan.

## References

[1] Gitlin D, Perricelli A, Gitlin GM. Synthesis of AFP by liver, yolk sac and intestinal tract of the human concepts. Cancer Res 1972;32:970–2.
[2] Belayew A, Tilghman SM. Genetic analysis of α-fetoprotein synthesis in mice. Mol Cell Biol 1982;2:1427–35.
[3] Chen H, Egan JO, Chiu JF. Regulation and activities of α-fetoprotein. Crit Rev Eukaryot Gene Expr 1997;7:11–41.
[4] Wu JT, Book L, Sudar K. Serum α-fetoprotein (AFP) levels in normal infants. Pediatr Res 1981;15:50–2.
[5] Ball D, Rose E, Alpert E. α-Fetoprotein levels in normal adults. Am J Med Sci 1992;303:157–9.
[6] Sato Y, Nakata K, Kato Y, et al. Early recognition of hepatocellular carcinoma based on altered profiles of α-fetoprotein. N Eng J Med 1993;328:1802–6.
[7] Lazarevich NL. Molecular mechanisms of α-fetoprotein gene expression. Biochemistry (Mosc) 2000;65:117–33.
[8] Jin DK, Vacher J, Feuerman MH. α-Fetoprotein gene sequences mediating Arf2 regulation during liver regeneration. Proc Natl Acad Sci USA 1998;95:8767–72.
[9] Sakurai T, Marusawa H, Satomura S, et al. Lens culinaris agglutinin-A-reactive α-fetoprotein as a marker for liver atrophy in fulminant hepatic failure. Hepatol Res 2003;26:98–105.
[10] Sakai M, Morinaga T, Urano K, Watanabe K, Wegmann TG, Tamaoki T. The human α-fetoprotein gene: sequence organization and the 5′-flanking region. J Biol Chem 1985;260:5055–60.
[11] Watanabe K, Saito A, Tamaoki T. Cell-specific silencer activity in a far upstream of the human α-fetoprotein gene. J Biol Chem 1987;262:4812–8.
[12] Sawadaishi K, Morinaga T, Tamaoki T. Interaction of a hepatoma-specific nuclear factor with transcriptional-regulatory sequences of the human α-fetoprotein and albumin gene. Mol Cell Biol 1988;8:5179–87.
[13] Nakabayashi H, Watanabe K, Saito A, Otsuru A, Sawadaishi K, Tamaoki T. Transcriptional regulation of α-fetoprotein expression by dexamethasone in human hepatoma cells. J Biol Chem 1989;264:266–71.
[14] Nakabayashi H, Hashimoto T, Miyao Y, Tjong KK, Chan J, Tamaoki T. A position-dependent silencer plays a major role in repressing α-fetoprotein expression in human hepatoma. Mol Cell Biol 1991;11:5885–93.
[15] Feuerman MH, Godbout R, Ingram RS, Tilghman SM. Tissue-specific transcription of the mouse α-fetoprotein gene promoter is dependent on HNF-1. Mol Cell Biol 1989;9:4204–12.
[16] Zhang DE, Hoyt PR, Papaconstantinou J. Localization of DNA protein-binding sites in the proximal and distal promoter regions of the mouse α-fetoprotein gene. J Biol Chem 1990;265:3382–91.
[17] Zhang DE, Xin G, Rabek JP, Papaconstantinou J. Functional analysis of the *trans*-acting factor binding sites of the mouse α-fetoprotein proximal promoter by site-directed mutagenesis. J Biol Chem 1991;266:21179–85.
[18] Bois-Joyeux B, Danan JL. Members of the CAAT/enhancer binding protein, hepatocyte nuclear factor-1 and nuclear factor-1 families can differentially modulate the activities of the rat α-fetoprotein promoter and enhancer. Biochem J 1994;301:49–55.
[19] McVey JH, Michaelides K, Hansen LP, et al. A G → A substitution in an HNF 1 binding site in the human α-fetoprotein gene is associated with hereditary persistence of α-fetoprotein (HPAFP). Hum Mol Genet 1993;2:379–84.
[20] Blesa JR, Ciner-Duran R, Vidal J, et al. Report of hereditary persistence of α-fetoprotein in a Spanish family: molecular basis and clinical concerns. J Hepatol 2003;38:541–4.
[21] Alj Y, Georgiakaki M, Savouret JF, et al. Hereditary persistence of α-fetoprotein is due to both proximal and distal hepatocyte nuclear factor-1 site mutations. Gastroenterology 2004;126:308–17.

[22] Thomassin H, Hamel D, Bernier D, Guertin M, Belanger L. Molecular cloning of two C/EBP-related proteins that bind to the promoter and the enhancer of the α1-fetoprotein gene: further analysis of C/EBPβ and C/EBPγ. Nucl Acids Res 1992;20:3091–8.
[23] Onaga Y, Ido A, Uto H, et al. Hypermethylation of the wild-type ferrochelatase allele is closely associated with severe liver complication in a family with erythropoietic protoporphyria. Biochem Biophys Res Commun 2004;321:851–8.
[24] Ido A, Nakata K, Kato Y, et al. Gene therapy for hepatoma cells using a retrovirus vector carrying herpes simplex virus thymidine kinase gene under the control of human α-fetoprotein promoter. Cancer Res 1995;55:3105–9.
[25] Ishikawa H, Nakata K, Mawatari F, et al. Utilization of variant-type of human α-fetoprotein promoter in gene therapy targeting for hepatocellular carcinoma. Gene Ther 1999;6:465–70.
[26] Liu F, Song YK, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. Gene Ther 1999;6:1258–66.
[27] Chevrette M, Guertin M, Turcotte B, Belanger L. The rat α1-fetoprotein gene: characterization of the 5′-flanking region and tandem organization with the albumin gene. Nucl Acids Res 1987;16:1338–9.
[28] Chouard T, Blumenfeld M, Bach I, Vandekerckhove J, Cereghini S, Yaniv M. A distal dimerization domain is essential for DNA-binding by the atypical HNF-1 homeodomain. Nucl Acids Res 1990;18:5853–63.
[29] Nicosia A, Monaci P, Tomei L, et al. A myosin-like dimerization helix and an extra-large homeodomain are essential elements of the tripartite DNA binding structure of LFB1. Cell 1990;61:1225–36.
[30] Yasuda H, Mizuno A, Tamaoki T, Morinaga T. ATBF1, a multiple homeodomain zinc finger protein, selectively down-regulates AT-rich elements of the human α-fetoprotein gene. Mol Cell Biol 1991;14:1395–401.
[31] Nakao K, Lawless D, Ohe Y, Miyao Y, Tamaoki T. c-Ha-ras down-regulates the α-fetoprotein gene but not the albumin gene in human hepatoma cells. Mol Cell Biol 1990;10:1461–9.
[32] Nakao K, Nakata K, Mitsuoka S, et al. Transforming growth factor b1 differentially regulates α-fetoprotein and albumin in HuH-7 human hepatoma cells. Biochem Biophys Res Commun 1991;174:1294–9.
[33] Nakata K, Motomura M, Nakabayashi H, Ido A, Tamaoki T. A possible mechanism of inverse developmental regulation of α-fetoprotein and albumin genes: studies with epidermal growth factor and phorbor ester. J Biol Chem 1992;267:1331–4.
[34] Rabek JP, Hoyt PR, Zhang DE, Izban MG, Papaconstantinou J. Derepression of a mouse α-fetoprotein expression vector in COS-1 cells by amplification of specific *cis*-acing sequences of the AFP promoter. Nucl Acids Res 1990;18:6677–82.
[35] Jose-Estanyol M, Danan JL. A liver-specific factor and nuclear factor I bind to the rat α-fetoprotein promoter. J Biol Chem 1988;263:10865–71.
[36] Bernier D, Thomassin H, Allard D, et al. Functional analysis of developmentally regulated chromatin-hypersensitive domains carrying the α1-fetoprotein gene promoter and the albumin/α1-fetoprotein intergenic enhancer. Mol Cell Biol 1993;13:1619–33.
[37] Coutois G, Baumhueter S, Crabtree GR. Purified hepatocyte nuclear factor 1 interacts with a family of hepatocyte-specific promoters. Proc Natl Acad Sci USA 1998;85:7937–41.
[38] Ueki T, Nakata K, Mawatari F, et al. Retrovirus-mediated gene therapy for human hepatocellular carcinoma transplanted in athymic mice. Int J Mol Med 1998;1:671–5.
[39] Uto H, Ido A, Hori T, et al. Hepatoma-specific gene therapy through retrovirus-mediated and targeted gene transfer using an adenovirus carrying the ecotropic receptor gene. Biochem Biophys Res Commun 1999;265:550–5.
[40] Ido A, Uto H, Moriuchi A, et al. Gene therapy targeting for hepatocellular carcinoma: selective and enhanced suicide gene expression regulated by a hypoxia-inducible enhancer linked to a human α-fetoprotein promoter. Cancer Res 2001;61:3016–21.

*Journal of Viral Hepatitis*, 2005, 12, 46–50 doi:10.1111/j.1365-2893.2005.00546.x

# The minimum number of clones necessary to sequence in order to obtain the maximum information about hepatitis C virus quasispecies: a comparison of subjects with and without liver cancer

G. Gao,[1] S. O. Stuver,[2,3] A. Okayama,[4] H. Tsubouchi,[4] N. E. Mueller[2] and E. Tabor[1] [1]*Division of Emerging and Transfusion Transmitted Diseases, Food and Drug Administration, Bethesda, MD, USA,* [2]*Department of Epidemiology, Harvard School of Public Health, Boston, MA, USA,* [3]*Department of Epidemiology, Boston University School of Public Health, Boston, MA, USA, and* [4]*Second Department of Internal Medicine, Miyazaki Medical College, Kiyotake, Miyazaki, Japan*

*Received May 2003; accepted for publication January 2004*

SUMMARY. Most studies of hepatitis C virus (HCV) quasispecies have reported the results of sequencing only three to five clones per sample. The possibility that sequencing so few clones might not provide a representative picture of the quasispecies present in a sample has never been evaluated. The present study was conducted to evaluate whether sequencing greater numbers of clones results in better information about the HCV quasispecies number and distribution, and to compare the HCV quasispecies in liver cancer cases and controls. RNA was extracted from serial serum samples from six subjects with HCV-associated liver cancer and 11 age- and sex-matched HCV-infected controls without liver cancer. The hypervariable region 1 (HVR1) of the HCV genome was amplified, cloned, and sequenced. For further studies of 12 serum samples from two liver cancer cases and two matched controls, successive groups of 10 additional clones were sequenced up to a total of 50 clones per serum sample. When only 10 clones were sequenced from each specimen, no consistent differences were seen between the number of HCV quasispecies in the six liver cancer cases and the 11 controls. However, sequencing 40 clones from each of 12 samples from two liver cancer cases and two controls revealed a greater number of quasispecies in liver cancer cases than in controls. Testing an additional 10 clones (50 clones per sample) did not significantly increase the number of quasispecies detected.

*Keywords*: hepatitis C virus, hepatocellular carcinoma, quasispecies.

## INTRODUCTION

One of the important characteristics of hepatitis C virus (HCV) is that its genome exhibits significant genetic heterogeneity as a result of the accumulation of mutations during viral replication. This high mutation rate, which is characteristic of RNA viruses, can be attributed to an error-prone RNA-dependent RNA polymerase that lacks proofreading activity. HCV, like other RNA viruses, circulates in an infected individual as a population of closely related, yet heterogeneous, quasispecies. The quasispecies population is generally composed of one dominant viral sequence (the master sequence) and a number of other sequences differing from the master sequence to various extents. The HCV hypervariable region 1 (HVR1) undergoes extensive variation during the course of chronic infection, and some of these changes may correspond to the emergence of immune-escape mutants, a proposed mechanism of HCV persistence.

In the present study, the genetic diversity of the HCV genome was evaluated in serum samples obtained prospectively in individuals with HCV infections who later developed liver cancer, and in matched control subjects. The impact of the number of clones sequenced on the number of quasispecies detected was further studied by sequential sampling of a large number of clones from selected serum samples.

Abbreviations: 5′-NCR, 5′-noncoding region; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HVR1, hypervariable region 1; PCR, polymerase chain reaction.

Correspondence: Edward Tabor M.D., Division of Emerging and Transfusion Transmitted Diseases, Office of Blood Research and Review, Food and Drug Administration, 1401 Rockville Pike, HFM-300, Rockville, MD 20852-1448, USA. E-mail: tabor@cber.fda.gov

## MATERIALS AND METHODS

### *Study subjects*

Serum samples were obtained from residents of one village in Miyazaki, Japan, who attended free, government-sponsored health examinations from November 1984 to November 2000. The prevalence of antibody to HCV (anti-HCV) in this village was 23% [1].

### *Serum samples*

Sequential serum samples were evaluated from six anti-HCV positive individuals who developed liver cancer between 1984 and 1999. Each of the six liver cancer cases was matched to two anti-HCV-positive controls by gender, age, and year of study enrollment, except for case no. 6, who was only matched with one control (a total of six cases and 11 controls). All 63 serum samples (23 from cases and 40 from controls) were coded. Samples had undergone fewer than two freeze–thaw cycles and had otherwise been kept at −80 °C until use.

### *Reverse transcription-polymerase chain reaction*

RNA was extracted from 0.25 mL of each serum sample and cDNA was synthesized. The 176 base pair (bp) HCV E2 fragment (containing the 81-nucleotide HVR1 and an additional 35 nucleotides upstream and 60 nucleotides downstream) and the 256-bp 5′-noncoding region (5′-NCR) fragment were amplified with gene-specific primers using nested polymerase chain reaction (PCR) (Table 1) [2].

To amplify the HVR1 fragment, the first round of PCR was performed with one sense primer [HCVHf1 (Table 1)] and a mixture of two antisense primers (HCVHb1 and HCVHb2); the second round of PCR was performed with one sense primer (HCVHf2) and a mixture of two antisense primers (HCVHb3 and HCVHb4). The amplification of the 5′-NCR fragment was carried out by conventional nested PCR using one sense (P-285) and one antisense primer (P-43) for the first round of PCR and one sense (P-276) and one antisense primer (P-50) for the second round of PCR [2]. The amplified HVR1 fragment was a 176-bp product from position +1366 to +1541 according to the numbering system of Choo *et al.* [3]. The amplified 5′-NCR fragment was a 256-bp product that extended from nucleotide position −276 to −21.

### *Cloning and sequencing of PCR products*

Polymerase chain reaction products were purified and cloned. Plasmid DNA was then extracted and sequenced on an automated DNA sequencer (model P310; PE/ABI, Foster City, CA, USA) with the BigDye Terminator Cycle Sequence Ready Reaction Kit (PE/ABI) using a universal M13 reverse primer according to protocols provided by the manufacturer. For most cases and controls, 10 clones from the HVR1 and five clones from the 5′-NCR were studied. However, for cases no. 3 and 5, and controls no. 3 and 5, 50 clones of the HVR1 fragment from each serum sample were amplified and sequenced in order to determine the extent to which examining additional clones would increase the number of quasispecies detected.

### *Sequence analysis*

The number of viral variants and the genetic diversity of the HCV quasispecies were assessed by examining viral sequences of the HVR1 and 5′-NCR genes. DNA sequence data were analysed with Edview software (PE/ABI).

**Table 1** Primers for amplifying HVR1 and 5′-NCR of HCV

| Name | Sequence | Positions |
|---|---|---|
| HVR1 – first PCR | | |
| HCVHf1 | 5′-GCC ATA TAA CGG GTC ACC GCA TGG C-3′ | 1208 to 1232 |
| HCVHb1 | 5′-CCC CAC GAC AAC AGG AC-3′ | 1811 to 1827 |
| HCVHb2 | 5′-TCC CAC CAC CAC GGG GC-3′ | 1811 to 1827 |
| HVR1 – second PCR | | |
| HCVHf2 | 5′-ATG GTG GGG AAC TGG GCG AAG G-3′ | 1366 to 1387 |
| HCVHb3 | 5′-ATG TGC CAA CTG CCG TTG GT-3′ | 1522 to 1541 |
| HCVHb4 | 5′-AGG TGC CAA CTG CCG TTG GT-3′ | 1522 to 1541 |
| 5′-NCR – first PCR | | |
| P-285 | 5′-ACT GTC TTC ACG CAG AAA GCG TCT AGC CAT-3′ | −285 to −256 |
| P-43 | 5′-CGA GAC CTC CCG GGG CAC TCG CAA GCA CCC-3′ | −14 to −43 |
| 5′-NCR – second PCR | | |
| P-276 | 5′-ACG CAG AAA GCG TCT AGC CAT GGC GTT AGT-3′ | −276 to −247 |
| P-50 | 5′-TCC CGG GGC ACT CGC AAG CAC CCT ATC AGG-3′ | −21 to −50 |

 

Sequence alignment and phylogenetic analysis were performed using MacVector V6.5 software (Oxford Molecular Group, Madison, WI, USA) and the GCG Wisconsin Package software (GCG, Madison, WI, USA). Genetic distance, a pairwise comparison of evolutionary distance between aligned sequences, was determined using the GCG Wisconsin Package software (GCG). For each serum sample (from the six cases and 11 controls), the DNA sequences from each of 10 clones of the PCR products were compared with the other nine clones (a total of 100 comparisons for each serum sample). The numerical result produced by the software reflects the number of substitutions per 100 nucleotides as well as the nature of the substitutions.

## RESULTS

The average genetic distance among 10 clones prepared from each serum sample was 18.3 among six cases (23 serum samples) and 6.7 among 11 controls (40 serum samples) ($P < 0.001$) (Fig. 1). Initially, 10 clones of HVR1 were sequenced from each serum sample. The number of quasispecies detected in each serum sample did not differ significantly between cases (average of 6.6 quasispecies) and controls (average of 5.1 quasispecies) when only 10 clones were sequenced from each sample (Fig. 2). In addition, no differences were seen in the number of quasispecies detected in early and late serum samples from the same individuals among both cases and controls when only 10 clones were sequenced (data not shown).

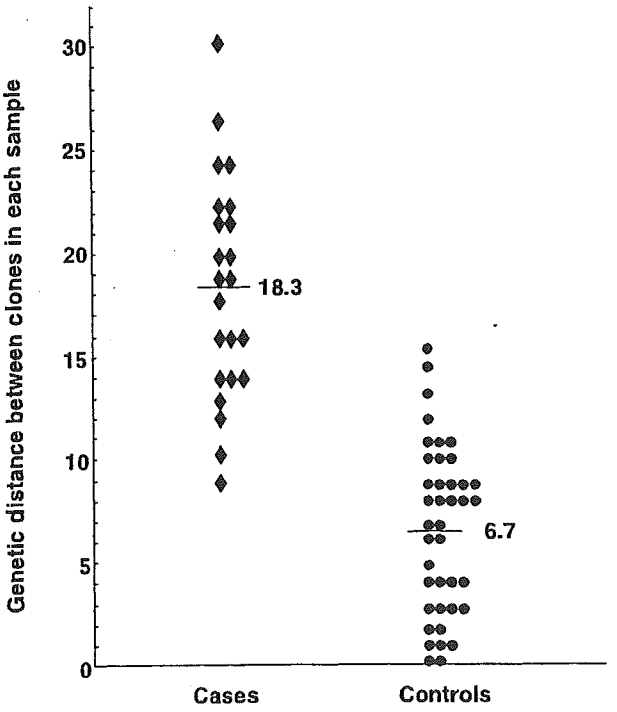


Fig. 1 Each mark represents the 'genetic distance' (see text for definition) between HCV RNA in the 10 clones from each of the serial serum samples from six liver cancer cases and 11 controls (63 serum samples total). Genetic distance, a pairwise comparison of evolutionary distance, was expressed as substitutions per 100 nucleotides. The average genetic distance within each serum specimen was 18.3 in cases and 6.7 in controls.

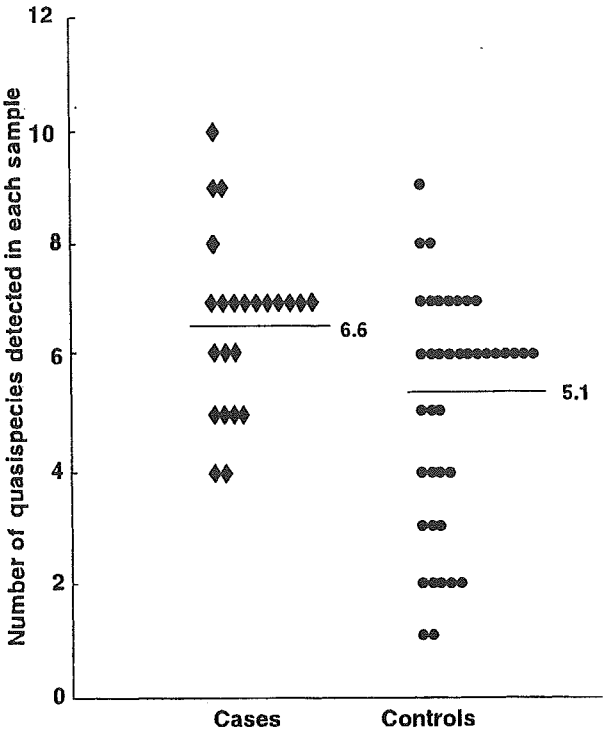


Fig. 2 Number of HCV quasispecies detected by sequencing 10 clones for each of 63 serum samples. There was no statistically significant difference between cases (average of 6.6 quasispecies) and controls (average of 5.1 quasispecies).

Forty additional clones each were analysed in sequential groups of 10 clones from cases no. 3 and 5 and their matched controls in samples obtained at the time of entry into the study, 1 year later, and 5 or 10 years later, for a total of 50 clones from each serum sample. After having sequenced 40 clones per sample, sequencing an additional 10 clones per sample detected only one or no additional quasispecies (Fig. 3).

During disease progression, the number of quasispecies detected by analysing 50 clones per sample in case no. 3 increased from 16 in 1984 to 22 in 1989, in control no. 3 from eight in 1984 to 15 in 1989, in case no. 5 from 18 in 1989 to 25 in 1994, and in control no. 5 increased from 6 in 1984 to 23 in 1994 (Fig. 3). The difference in the number of quasispecies between these cases and controls at the time of entry into the study (1984, average of 17 quasispecies in cases and seven in controls) was higher than 5–10 years later (1989 or 1994, average of 23.5 quasispecies in cases and 19 in controls).

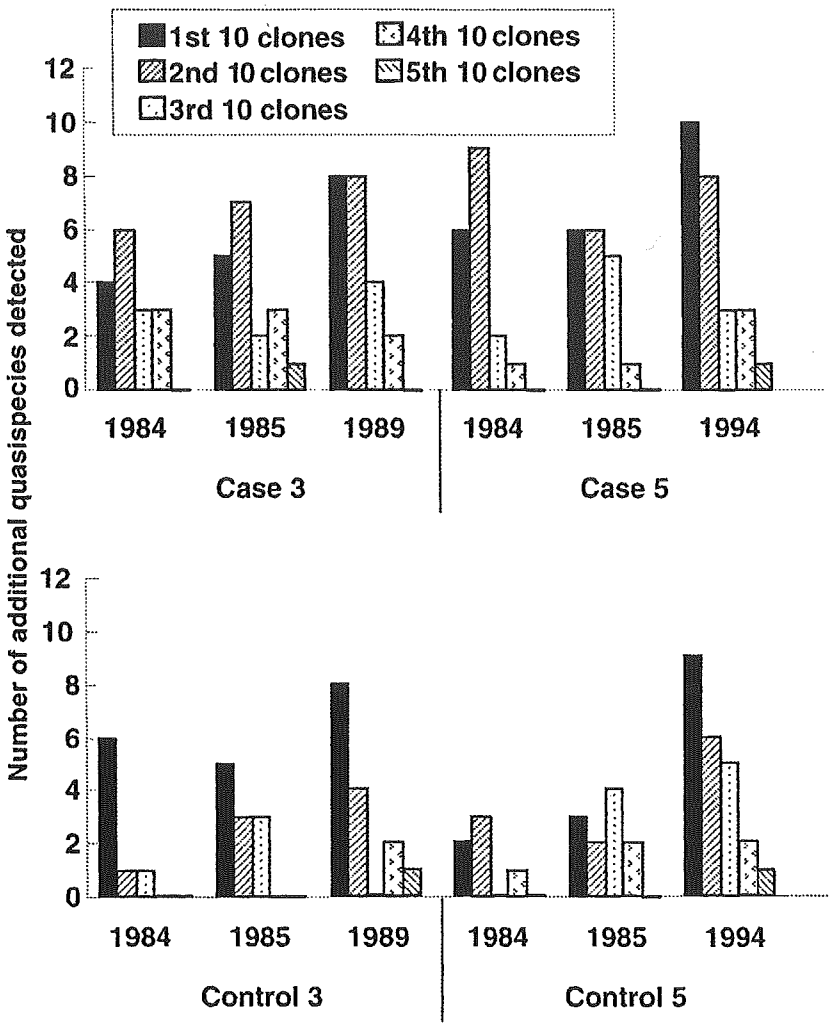


Fig. 3 Correlation between the number of clones sequenced and the number of *additional* quasispecies detected. Fifty clones were sequentially analysed in incremental groups of 10 clones each from cases no. 3 and 5 and controls no. 3 and 5, using serum samples obtained at entry to the study, 1 year later, and 5 or 10 years later. The bars indicate the number of *additional* quasispecies detected among those 10 clones (over and above those detected by testing prior groups of 10 clones each). The ability to detect additional quasispecies decreased as more clones were analysed. Nearly all detectable quasispecies were detected in the first 40 clones (i.e. one or no additional quasispecies were detected by testing 50 clones, compared with testing 40 clones).

## DISCUSSION

The quasispecies nature of HCV is thought to play an important role in maintaining and modulating viral replication [4,5], and the increasing diversity of the quasispecies in one individual is associated with more advanced liver damage caused by HCV [6]. In the present study, the quasispecies diversity in those who subsequently developed hepatocellular carcinoma (HCC) was greater than that in controls (average genetic distance 18.3 in cases and 6.7 in controls).

In the present study, the number of quasispecies was seen to increase dramatically over 5–10 years in four patients and controls from whom 50 clones per sample were studied, reaching a level between 15 and 25 quasispecies per serum sample. However, overall sample-to-sample fluctuation was seen for each of the other seven patients and controls, from whom only 10 clones per sample were studied.

The present study shows the importance of sequencing a large number of clones in order to obtain the most accurate assessment of quasispecies diversity. Most published studies of HCV quasispecies have reported the results of sequencing only three to five clones per sample. However, Torres-Puente *et al.* [7] recently reported that no significant difference was observed in genetic variability tested in small numbers of samples (10 sequences) compared with large numbers (100 sequences). In contrast, the present study shows that sequencing a small number of clones per sample provides less information about quasispecies diversity than sequencing 20, 30, or 40 clones per sample. The data in this study also suggest that the maximum number of quasispecies in a serum sample usually can be detected by sequencing about 40 clones per sample.

Obtaining an accurate picture of the quasispecies diversity in the serum of individuals infected with HCV may lead to a better understanding of the pathogenesis. There may be differences in virulence among quasispecies; some of the quasispecies in an individual are not transmitted in at least some cases of maternal–fetal transmission [8], needlestick accidents [2], or experimental transmission to chimpanzees [9]. Theoretically, some quasispecies might bind to cellular receptors more readily or might escape the host immune

response more easily. The association of quasispecies diversity with the emergence of escape mutants [10], and the correlation of greater HCV quasispecies diversity with more severe liver damage [11], indicate that quasispecies diversity plays an important role in the course of HCV infection.

## REFERENCES


1 Okayama A, Stuver SO, Tabor E *et al.* Incident hepatitis C virus infection in a community-based population in Japan. *J Viral Hepat* 2002; 9: 43–51.

2 Gao G, Buskell Z, Seeff L *et al.* Drift in the hypervariable region of the hepatitis C virus during 27 years in two patients. *J Med Virol* 2002; 68: 60–67.

3 Choo Q-L, Richman KH, Han JH *et al.* Genetic organization and diversity of the hepatitis C virus. *Proc Natl Acad Sci USA* 1991; 88: 2451–2455.

4 Domingo E, Holland JJ. RNA virus mutations and fitness for survival. *Annu Rev Microbiol* 1997; 51: 151–178.

5 Korenaga M, Hino K, Katoh Y *et al.* A possible role of hypervariable region 1 quasispecies in escape of hepatitis C virus particles from neutralization. *J Viral Hepat* 2001; 8: 331–340.

6 Koizumi K, Enomoto N, Kurosaki M *et al.* Diversity of quasispecies in various disease stages of chronic hepatitis C virus infection and its significance in interferon treatment. *Hepatology* 1995; 22: 30–35.

7 Torres-Puente M, Bracho MA, Jimenez N, Garcia-Robles I, Moya M, Gonzalez-Candelas F. Sampling and repeatability in the evaluation of hepatitis C virus genetic variability. *J General Virol* 2003; 84: 2343–2350.

8 Weiner AJ, Thaler MM, Crawford K *et al.* A unique, predominant hepatitis C virus variant found in an infant born to a mother with multiple variants. *J Virol* 1993; 67: 4365–4368.

9 Sugitani M, Shikata T. Comparison of amino acid sequences in hypervariable region-1 of hepatitis C virus clones between human inocula and the infected chimpanzee sera. *Virus Res* 1998; 56: 177–182.

10 Farci P, Bukh J, Purcell RH. The quasispecies of hepatitis C virus and the host immune response. *Springer Semin Immunopathol* 1997; 19: 5–26.

11 Duffy M, Salemi M, Sheehy M *et al.* Comparative rates of nucleotide sequence variation in the hypervariable region of E1/E2 and the NS5b region of hepatitis C virus in patients with a spectrum of liver disease resulting from a common source of infection. *Virology* 2002; 301: 354–364.

*Journal of Viral Hepatitis*, 2005, **12**, 106–110 doi:10.1111/j.1365-2893.2005.00558.x

# Usefulness of a new immuno-radiometric assay to detect hepatitis C core antigen in a community-based population

K. Hayashi,[1] S. Hasuike,[1] K. Kusumoto,[1] A. Ido,[2] H. Uto,[1] N. Kenji,[1] M. Kohara,[3] S. O. Stuver[4,5] and H. Tsubouchi[1] [1]*Department of Internal Medicine II, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan;* [2]*Translational Research Center, Kyoto University, Kyoto, Japan;* [3]*Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan;* [4]*Department of Epidemiology, Boston University School of Public Health, Boston, MA; and* [5]*Department of Epidemiology, Harvard School of Public Health, Boston, MA, USA*

*Received November 2003; accepted for publication March 2004*

SUMMARY. A new immuno-radiometric assay (IRMA) to detect hepatitis C virus (HCV) core antigen (HCVcAg) has been developed. The aim of the present study was to investigate the sensitivity and specificity of this IRMA to measure HCV antigenemia, based on the detection of HCV RNA as the gold standard, and to assess the utility of the IRMA in a community-based population. Anti-HCV positive residents in a hyperendemic area of HCV infection in Japan were studied. Serum levels of HCVcAg were measured using IRMA, and the presence of HCV RNA was determined by a qualitative reverse transcription-polymerase chain reaction (RT-PCR) assay. The sensitivity and the specificity of the IRMA were 96.4 and 100%, respectively. The sensitivity of the IRMA was similar between serological HCV group I (HCV genotypes 1a and 1b) (97.6%) and group II (HCV genotypes 2a and 2b) (94.0%). There was a strong correlation between serum HCVcAg level and HCV-RNA measured by a quantitative RT-PCR ($r = 0.832$, $P < 0.0001$). There also was a very strong correlation of HCVcAg level between IRMA measurements performed on serum and those performed on plasma ($r = 0.984$, $P < 0.0001$). In conclusion, this new IRMA is useful for the detection of HCV core antigen in a community-based population.

*Keywords*: community-based population, hepatitis C virus core antigen, immuno-radiometric assay, serological hepatitis C virus group.

## INTRODUCTION

Persistent infection with hepatitis C virus (HCV), as well as hepatitis B virus (HBV), is a primary cause of chronic liver disease, such as chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC). In Town C of Miyazaki Prefecture in Japan, the average annual mortality from liver disease was estimated to be 102.5/100 000 between 1995 and 1997, which was substantially higher than the estimate of 37.6/100 000 for all of the prefecture, as reported by the prefectural public health service. In 1993, over 4000 residents of Town C were tested for antibody to HCV (anti-HCV) and HBV surface antigen (HBsAg) in conjunction with the local, government-sponsored general health examination conducted in the town. The prevalence of anti-HCV positivity was found to be 22.5% and that of HBsAg positivity, 1.1%. Thus, the elevated mortality from liver disease in Town C is considered to be due to the high prevalence of HCV infection in this population.

Measurement of anti-HCV is generally used to screen for HCV infection. However, not all anti-HCV positive people are persistently infected with the virus. Therefore, a simple, quick, and inexpensive method for measuring HCV viremia is required for population-based testing. Although the detection of HCV RNA by reverse transcription-polymerase chain reaction (RT-PCR) represents the most sensitive method for determining persistent HCV infection, the assay is time-consuming, costly, and technically demanding. In contrast, enzyme immunoassays (EIAs) to detect HCV core antigen (HCVcAg) are simple and relatively inexpensive [1]. A number of reports have demonstrated the utility of measuring HCVcAg using EIAs [2–5]. Recently, a new immuno-radiometric assay (IRMA) to detect HCVcAg was developed.

The aim of the present study was to investigate the sensitivity and specificity of this new IRMA to measure HCV antigenemia, based on the detection of HCV RNA as the gold

Abbreviation: EIA, enzyme immunoassay; HCC, hepatocellular carcinoma; IRMA, immuno-radiometric assay; RT-PCR, reverse transcription-polymerase chain reaction; US, ultrasonography.

Correspondence: Katsuhiro Hayashi, 5,200 Kihara, Kiyotake, Miyazaki 889-1692, Japan. E-mail: katuhaya@med.miyazaki-u.ac.jp

standard, and to assess the utility of the IRMA in a community-based population such as Town C.

### *Methods and subjects*

Since 1994, 1083 Town C residents have been identified as being positive for anti-HCV and have been screened annually for the early detection of HCC by ultrasonography (US) examination. To elucidate the natural history of HCV infection and the risk factors for HCC, additional virologic, epidemiologic, and clinical data also have been collected at these US examinations, beginning in 2001. Blood samples for CBC, liver function test, tumour markers (alpha-fetoprotein and PIVKA-II), hepatic fibrosis markers (hyaluronic acid and type IV collagen) and HCV-associated markers (anti-HCV antibody titre, serological genotyping of HCV, virus load, presence of HCV RNA) are collected after taking an informed consent. In 2002, 931 subjects were invited to the annual US examination, of whom 674 (72%) attended and provided a blood sample.

### *Anti-HCV antibody*

Anti-HCV antibody was measured by chemiluminescent enzyme immunoassay using a third generation HCV antibody kit (Lumipulse Ortho II; Ortho-Clinical Diagonostics K. K., Tokyo, Japan).

### *HCV antigen load*

At the 2002 US screening, the new IRMA (Ortho HCV Ag IRMA Test; Ortho-Clinical Diagnostics K. K.) was used to detect HCVcAg. Testing using the IRMA was carried out as indicated by the manufacturer. Briefly, serum or standard sample was added to pretreatment solution in a plastic tube and incubated at 56–60 °C. After adding the reaction solution and one bead coated with two different monoclonal antibodies against HCVcAg to each tube, the mixture was incubated at room temperature. Two different horse-radish peroxidase-conjugated monoclonal antibodies against HCVc Ag was added to each tube. $^{125}$I-conjugated polyclonal antibodies against peroxidase then were added, and the radioactivity of the sample and the standard was measured with a $\gamma$-scintillation counter. The concentration of HCVcAg was expressed as femto-mol/L (fmol/L). The range of the IRMA was from 20 to 20 000 fmol/L. When the titre of HCVcAg was less than 20 fmol/L, the sample was judged as negative. The plasma levels of HCVcAg also were measured, by the same IRMA method, for a subset of 40 subjects.

### *HCV RNA detection and quantification*

The presence of HCV RNA in serum was determined by a qualitative RT-PCR assay kit (Amplicore® HCV; Nippon Roche, Tokyo, Japan). The detectable HCV-RNA by this assay kit was 10 copy/mL. The serum HCV RNA level was measured using a quantitative RT-PCR assay kit (Amplicore® GT HCV Monitor v2.0; Nippon Roche) on a random sample of 100 HCV RNA positive subjects. However, four of the subjects had an insufficient sample for measuring HCV RNA level. The concentration of HCV RNA was expressed as KIU/mL, and the range was from 0.5 to 850 KIU/mL. When the titres of HCV-RNA were less than 0.5 KIU/mL, the sample was judged as negative.

### *Serological HCV group*

Serological HCV groups were determined by a serological genotyping assay (Immunocheck F-HCV Grouping; International Reagents Co., Kobe, Japan) [6]. When the serological group could not be clearly classified by this assay, HCV genotypes were determined by the RT-PCR method [7]. Genotypes 1a and 1b were defined as serological HCV group I, and genotypes 2a and 2b as group II.

## RESULTS

A total of 613 (90.9%) of the 674 residents who attended the 2002 US screening and provided a blood sample were anti-HCV antibody positive and 61 were negative. Thirty-eight per cent of the subjects studied were men ($n$ = 233), and the overall mean age was 69.3 years.

The results of the IRMA testing are summarized in Table 1. HCVcAg was detected by IRMA in 424 (69.2%) of the 613 anti-HCV positive subjects; HCV RNA was detected by RT-PCR in 440 (71.8%). Based on the HCV RNA status as the gold standard, the sensitivity of the IRMA was 96.4% (424/440), with a specificity of 100%. All 61 subjects who were anti-HCV negative were negative for both HCVcAg and HCV-RNA. Ninety-six randomly selected serum samples were tested for HCV-RNA level using a quantitative RT-PCR assay (Table 2). Neither HCVcAg by IRMA nor HCV-RNA by RT-PCR was detected in four samples. In three samples, HCV-RNA was detected only by RT-PCR. The HCV-RNA level of these samples was 1.3, 7.1 and 19 KIU/mL. There were no samples that were HCVcAg positive but negative for HCV-RNA (Tables 1 and 2). Figure 1 shows a strong correlation

**Table 1** Comparison of the detection of HCV core antigen by immuno-radiometric assay with the detection of HCV-RNA by RT-PCR

| | HCV-RNA by qualitative RT-PCR | |
|---|---|---|
| HCVcAg by IRMA | + | – |
| + | 424 | 0 |
| – | 16 | 173 |

HCV, hepatitis C virus; RT-PCR, reverse transcription-polymerase chain reaction.

**Table 2** Comparison of the detection of HCV core antigen by IRMA and HCV-RNA by quantitative RT-PCR in HCV-RNA positive subjects

| HCVcAg by IRMA | HCV-RNA by quantitative RT-PCR | |
|---|---|---|
| | + | − |
| + | 89 | 0 |
| − | 3 | 4 |

HCV, hepatitis C virus; IRMA, immuno-radiometric assay; RT-PCR, reverse transcription-polymerase chain reaction.

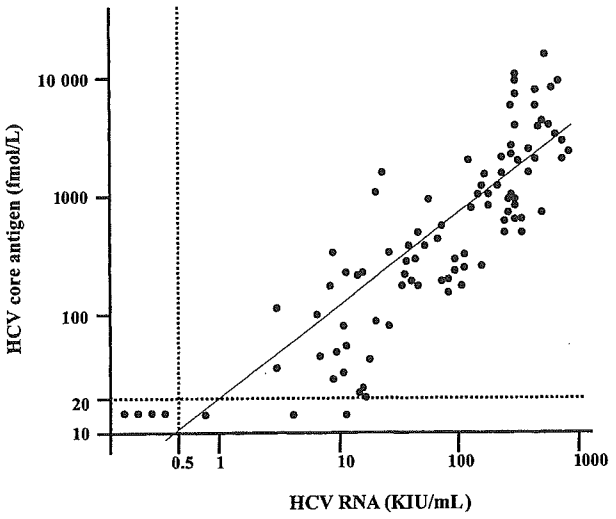


**Fig. 1** Correlation between concentration of HCV RNA measured by AMPLICOR Monitor test and HCV core antigen measured by immuno-radiometric assay. The serum HCV RNA level was measured using a quantitative RT-PCR assay kit (Amplicore® GT HCV Monitor v2.0) on a random sample of ninety-six HCV RNA positive subjects. The levels of serum HCVcAg measured by IRMA were strongly correlated with HCV-RNA levels by RT-PCR (Pearson correlation coefficient, $r = 0.832$, $P < 0.0001$).

between serum HCVcAg level measured by IRMA and HCV-RNA level by RT-PCR ($r = 0.832$, $P < 0.0001$).

The HCV genotype group could be determined by serological genotyping or RT-PCR assay for 524 subjects who attended the 2002 screening (Table 3). The distribution of subjects by HCV genotype group was 356 (67.9%) of the 524 subjects in group I and 168 (32.1%) in group II. The sensitivity of the IRMA was similar in the two serological HCV genotype groups (97.6% in group I and 94.0% in group II) (Table 3). It is noteworthy that the average amount of HCVcAg detected by the IRMA was similar in the two-genotype groups (median of 3060 fmol/L in group I and median of 3350 fmol/L in group II).

The correlation between serum and plasma levels of HCVcAg detected by IRMA was analysed in a subset of the Town C residents who attended the 2002 US screening (Fig. 2). The levels of HCVcAg in plasma were strongly correlated with those in serum (Pearson correlation coefficient, $r = 0.984$, $P < 0.0001$).

**Table 3** Comparison of the detection of HCV core antigen by IRMA with the detection of HCV RNA by RT-PCR, by serological HCV group

| HCVcAg by IRMA | HCV-RNA by RT-PCR | |
|---|---|---|
| | + | − |
| Serological HCV group I | | |
| + | 282 | 0 |
| − | 7 | 67 |
| Serological HCV group II | | |
| + | 141 | 0 |
| − | 9 | 18 |

HCV, hepatitis C virus; IRMA, immuno-radiometric assay; RT-PCR, reverse transcription-polymerase chain reaction.

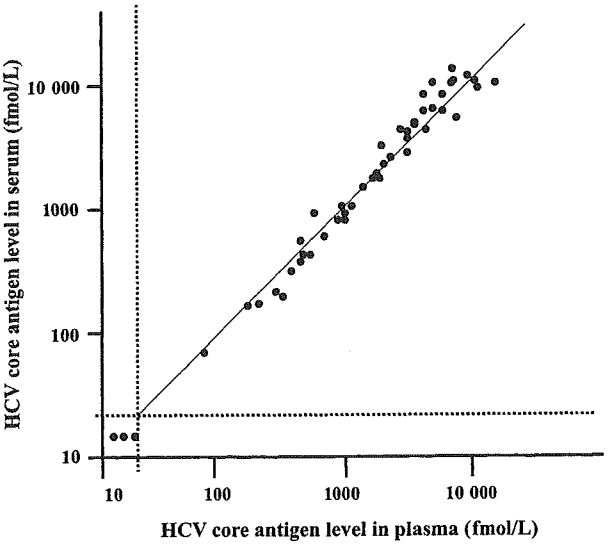


**Fig. 2** Correlation between HCV core antigen levels in serum and in plasma measured by immuno-radiometric assay. The subjects were classified into six groups according to their serum HCVcAg levels (<20, 20–100, 100–1,000, 1000–10 000, 10 000–20 000, >20 000 fmol/L). Plasma samples from five or six subjects were randomly selected from each group for a total of 40 subjects and were measured by IRMA. The levels of HCVcAg in plasma were strongly correlated with those in serum (Pearson correlation coefficient, $r = 0.984$, $P < 0.0001$).

## DISCUSSION

HCV core antigen was first detected in the circulation of HCV-infected hosts using EIA-based methods [1,8,9]. Many

reports have demonstrated the utility of EIA to detect HCVcAg for monitoring the effect of interferon therapy [1], for following liver transplantation patients with HCV recurrence [2], for quantitative evaluation of HCV viremia in anti-HCV positive patients [3], and as a marker of HCV viremia in the serological window-phase period [4]. However, the first versions of the EIA for HCVcAg had some limitations. They could not detect HCVcAg below a level of 20 KIU/mL of HCV RNA, so that their use was limited to the monitoring of late events during and after antiviral treatment [10]. In addition, the pretreatment process of the sample was somewhat complicated, with three steps required to expose the epitopes of HCVcAg bound by low-density lipoprotein or anti-HCV core antibody. The sensitivity of the EIA also was affected by mutations in the HCV core region [11]. Moreover, differences in the detectable levels of HCVcAg titres between the serological HCV genotype groups resulted in a lower sensitivity of the EIA among people infected with HCV genotype 2 [12].

In 1999, Aoyagi and colleagues developed a modified version of the EIA for HCVcAg [5]. In this version, the epitope of HCVcAg could be easily exposed, and binding by anti-HCV core antibody in the serum could be reduced by incubation with three types of detergents. Since the modified EIA required only one pretreatment step, it was simpler than the first generation versions of the assay. In addition, it had a 100-fold increase in sensitivity over the earlier versions. Tanaka *et al.* demonstrated that the second generation of the EIA for HCVcAg was useful for the diagnosis of acute and chronic hepatitis C and for predicting and monitoring the effect of interferon treatment [13]. More recently, a new IRMA-based test for detecting HCVcAg, which is a further modification of the Aoyagi *et al.* EIA method, was developed.

In the present study, we investigated the sensitivity and specificity of this IRMA, to detect HCV persistent infection based on the presence of HCV RNA as the gold standard. The sensitivity of the IRMA was 96.4%. In contrast, we tested for HCVcAg by a first generation assay at the 2001 US screening and found its sensitivity was 85.4% (data not shown). Of the randomly selected 96 samples, there was a strong correlation between serum HCVcAg level measured by the IRMA and HCV-RNA level by a commercial quantitative RT-PCR assay. This result is the same as that previously reported by Tanaka *et al.* [13]. Moreover, the IRMA for HCVcAg overcomes the problem of the effect of serological HCV genotype group on the level of HCVcAg detectable by EIA in serum [12]. In our population, the sensitivity of the first generation EIA was significantly lower in serological group II (HCV genotypes 2a and 2b) (74.7%) than in group I (HCV genotypes 1a and 1b) (90.5%) (data not shown). In striking contrast, the sensitivity of the IRMA was 94% or higher in both serological HCV genotype groups. It is not clear why the sensitivity of the IRMA is not affected by HCV genotype group. Both the EIA [1] and the IRMA [5] use high-affinity monoclonal antibodies that recognize amino acid sequences known to be relatively well conserved across the six HCV genotypes. However, small differences in these amino acid sequences between genotype groups I and II may exist, which render the monoclonal antibodies in the first generation EIA less sensitive to detect genotype group II core antigen. Moreover, the four different monoclonal antibodies used in the second generation IRMA may enhance the ability of this assay to detect HCVcAg in persons infected with HCV group II genotypes.

The presence of HCV RNA is considered the gold standard for determining HCV persistence, and RT-PCR is a very sensitive method to detect HCV RNA. However, the RT-PCR assay requires additional amplification procedures and specially-trained personnel and carries the risk of contamination. In addition, it has been reported that the RT-PCR method produces false negative results for heparinized samples [14] and may not detect the lower HCV RNA levels found for persons infected with HCV genotypes 2a or 2b [15]. There also is a progressive and significant loss of HCV RNA activity when the time from the formation of the clot until centrifugation is longer than 2 h from the collection of the blood specimen [16]. In contrast, EIA-based methods to detect HCVcAg offer several advantages over RT-PCR for measuring HCV persistence. First, EIAs are not influenced by the anticoagulants EDTA, heparin, or sodium citrate [5]. In the current study, there was a very strong correlation between HCVcAg levels in serum and those in plasma as measured by the new IRMA. Second since the HCVcAg detected by EIA is stable [5], extra precautions in processing and storing specimens also should not be necessary. Moreover, the IRMA appears to be sufficiently sensitive to identify persistent group II HCV infection. Finally, the cost of the IRMA kit is less than one-third that of the RT-PCR assay.

The majority of HCV carriers are asymptomatic, and some may display advanced liver disease, including liver cirrhosis and HCC, without the awareness that they are infected with HCV. Thus it is important that persons with persistent HCV infection can be identified. In the present study, we demonstrated the usefulness of a new IRMA for the detection of HCV antigenemia in the community-based Town C population in Japan. The assay is relatively simple and inexpensive and has a high sensitivity to detect HCVcAg in people infected with HCV genotypes 1 or 2. Thus, this new IRMA is an economically viable option for identifying individuals with chronic HCV infection when screening large numbers of people on a population level.

## ACKNOWLEDGEMENTS

This work was supported by a grant (no. CA87982) from the United States National Institutes of Health and by a grant-in-aid (Research on Hepatitis and BSE) from the Ministry of Health, Labour and Welfare, Japan.

## REFERENCES


1 Tanaka T, Lau JYN, Mizokami M *et al.* Simple fluorescent enzyme immunoassay for detection and quantification of hepatitis C viremia. *J Hepatol* 1995; 23: 742–745.

2 Dickson RC, Mizokami M, Orito E, Qian KP, Lau JY. Quantification of serum HCV core antigen by a fluorescent enzyme immunoassay in liver transplant recipients with recurrent hepatitis C – clinical and virologic implications. *Transplantation* 1999; 68: 1512–1516.

3 Komatsu F, Takahashi K. Determination of serum hepatitis C (HCV) core protein using a novel approach for quantitative evaluation of HCV viremia in anti-HCV-positive patients. *Liver* 1999; 19: 375–380.

4 Widell A, Molnegren V, Pieksma F, Calmann M, Peterson J, Lee SR. Detection of hepatitis C core antigen in serum or plasma as a marker of hepatitis C viremia in the serological window-phase. *Transfusion* 2002; 12: 107–113.

5 Aoyagi K, Ohue C, Iida K *et al.* Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. *J Clin Microbiol* 1999; 37: 1802–1808.

6 Tsukiyama-Kohara K, Yamaguchi K, Maki N *et al.* Antigenicities of grouping I and II hepatitis C virus polypeptides – molecular basis of diagnosis. *Virology* 1993; 192: 430–437.

7 Ohno T, Mizokami M, Wu RR *et al.* 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: 201–207.

8 Takahashi K, Okamoto H, Kishimoto S *et al.* Demonstration of a hepatitis C virus-specific antigen predicted from the putative core gene in the circulation of infected hosts. *J Gen Virol* 1992; 73: 667–672.

9 Kashiwakuma T, Hasegawa A, Kajita T *et al.* Detection of hepatitis C virus specific core protein in serum of patients by a sensitive fluorescence enzyme immunoassay (FEIA). *J Immunol Methods* 1996; 190: 79–89.

10 Bouvier-Alias M, Patel K, Dahari H *et al.* Clinical utility of total HCV core antigen quantification: a new indirect marker of HCV replication. *Hepatol* 2002; 36: 211–218.

11 Tokita H, Kaufman G, Matsubayashi M *et al.* Hepatitis C virus core mutations reduce the sensitivity of a fluorescence enzyme immunoassay. *J Clin Microbiol* 2000; 38: 3450–3452.

12 Orito E, Mizokami M, Tanaka T *et al.* Quantification of serum hepatitis C virus core protein level in patients chronically infected with different hepatitis C virus genotypes. *Gut* 1996; 39: 876–880.

13 Tanaka E, Ohue C, Aoyagi K *et al.* Evaluation of a new enzyme immunoassay for hepatitis C virus (HCV) core antigen with clinical sensitivity approximating that of genomic amplification of HCV RNA. *Hepatology* 2000; 32: 388–393.

14 Willems M, Moshage H, Nevens F, Fevery J, Yap SH. Plasma collected from heparinized blood is not suitable for HCV-RNA detection by conventional RT-PCR assay. *J Virol Methods* 1993; 42: 127–130.

15 Davis GL, Lau YYN, Urdea MS *et al.* Quantitative detection of hepatitis C virus RNA with a solid-phase signal amplification method: detection of optimal conditions for specimen collection and clinical application in interferon-treated patients. *Hepatology* 1994; 19: 1337–1341.

16 Hawkins A, Davidson F, Simmonds P. Comparison of plasma virus loads among individuals infected with hepatitis C virus (HCV) genotype 1, 2, and 3 by Quantiplex HCV assay versions 1 and 2, Roche Monitor assay, and in-house limiting dilution methods. *J Clin Microbiol* 1997; 35: 187–192.

最新医学・第60巻・第4号　(2005年4月号 別刷)

特集　肝疾患研究の新たな展開

# HGFによる劇症肝炎の治療

## —トランスレーショナルリサーチの現況—

井戸章雄　森内昭博　金　一徳
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最新医学社

# HGFによる劇症肝炎の治療

## —トランスレーショナルリサーチの現況—

井戸章雄[**1]　森内昭博[*1]　金　一徳[*1]
宇都浩文[*2]　坪内博仁[***1**2]

要　旨

HGFは，肝再生を促進しアポトーシスを抑制することから，治療薬としての臨床応用が期待されている．現在，筆者らは劇症肝炎および遅発性肝不全を対象とした「組換えヒトHGF」の臨床試験の準備を進めているが，安全性を確保すること，倫理的に承認された臨床プロトコールを作成し客観的な評価に耐えうるデータを得ることが極めて重要である．

### はじめに

肝細胞増殖因子（HGF）は筆者らによって単離・精製された増殖因子で，肝細胞のみならず種々の上皮系細胞，内皮細胞および一部の間葉系細胞に対して，増殖促進，細胞遊走促進，形態形成，抗アポトーシスなど多彩な作用を誘導する[1~4]．筆者らは，これまでに製薬会社と共同して組換えヒトHGFの医薬品化を進め，人体へ投与可能な精製レベルの組換えヒトHGFが供給される見通しとなった．このような背景から，劇症肝炎，肝移植，肝硬変を対象としたトランスレーショナルリサーチ「HGF肝再生医療プロジェクト」（リーダー　坪内博仁　京都大学大学院　客員教授）が，京都大学医学部附属病院探索医療センター探索医療開発部において2002年7月からスタートした．一方，これまで人体に投与されたことのない組換えヒトHGFの臨床応用は，当初から救命率約25%と予後不良の劇症肝炎および遅発性肝不全を対象とした第Ⅰ・Ⅱ相臨床試験として実施すべく準備を進めている．本稿では，トランスレーショナルリサーチとして進められている組換えヒトHGFの臨床応用について，その基礎的データから安全性の問題点と対応，臨床試験の倫理的妥当性について述べる．

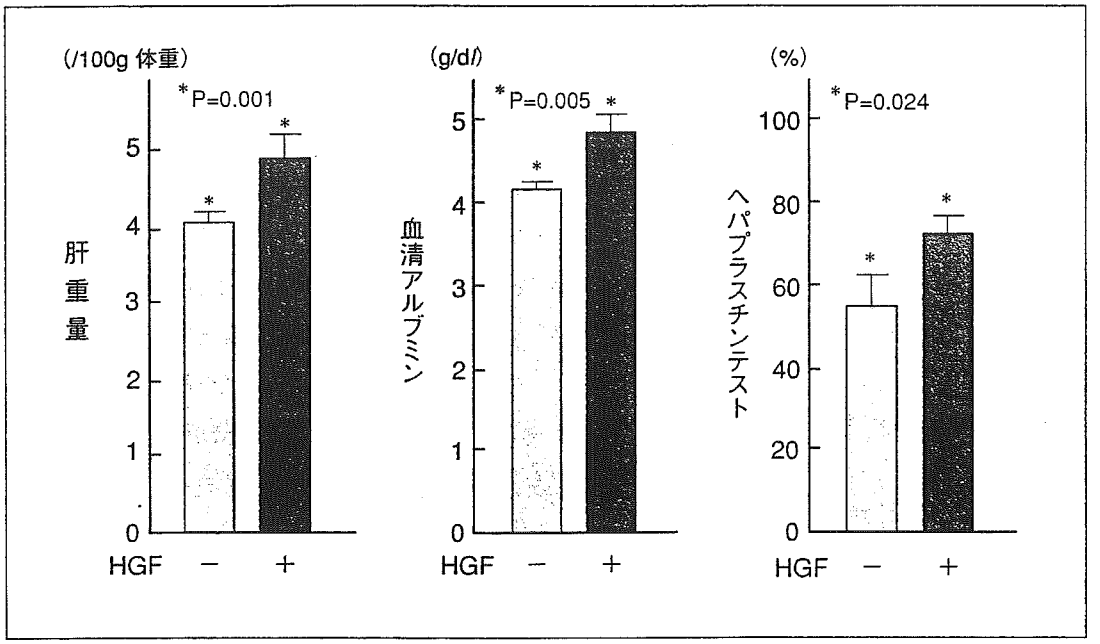
### 劇症肝炎において期待されるHGFの作用

劇症肝炎は，急激に起こる肝の広範性壊死に基づいて意識障害（肝性脳症）を主徴とする急性肝不全症状を呈する疾患である．劇症肝炎患者血清中のHGFは著しく上昇し，血

[*1] 京都大学医学部附属病院　探索医療センター　探索医療開発部　[**1] 同　助教授　[***1] 同　客員教授
[*2] 宮崎大学医学部　内科学第二講座　講師　[**2] 同　教授

キーワード：HGF，劇症肝炎，肝再生，抗アポトーシス，非臨床試験

図1　ジメチルニトロソアミン（DMN）誘導肝障害モデルにおける組換えヒトHGFの肝再生促進作用



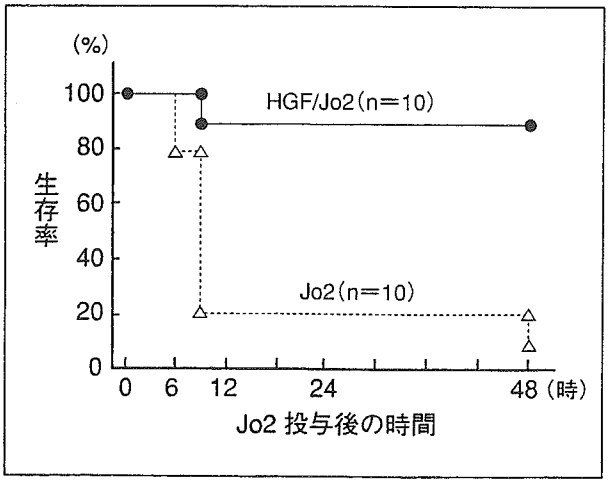

DMN 2 mg/kg を2日間腹腔内投与して誘導した肝障害モデルに10日間組換えヒトHGF（1 mg/kg）を静脈内投与したところ，肝重量および血清アルブミンが増加し，ヘパプラスチンテストが改善した．

清 HGF 測定は劇症肝炎の早期診断や予後予測に有用とされている[5~7]．この血清 HGF 上昇は，広範な肝壊死に伴って標的細胞である肝細胞が減少し，また肝組織における HGF の産生が増加したためと考えられる．血清 HGF が上昇しているにもかかわらず肝再生不全となっている機序は，劇症肝炎患者の肝生検組織の採取が難しいことから十分解明されていない．肝移植が施行された劇症肝炎患者の肝組織を用いた解析では，HGF の発現は著しく亢進していることが確認されており，c-Met については相反する成績が報告されている[8][9]．また，TGFβ 発現亢進が肝細胞増殖抑制に関与することも示唆されている[10]．いずれにしても，劇症肝炎における肝再生不全のメカニズムはいまだ不明の点が多いが，劇症肝炎患者における肝再生不全には HGF などの肝再生因子と TGFβ などの肝再生抑制因子のバランスが関与していることが考えられる．したがって，外因性 HGF がこの肝再生因子/抑制因子のバランスを肝再生側に傾けて効果を発揮する可能性が十分期待される．実際，ジメチルニトロソアミン（DMN）によって誘導した肝障害ラットでも内因性の HGF が上昇しているが，10 日間組換えヒト HGF を投与すると肝重量および血清アルブミンが有意に増加し，肝予備能も改善する（図1）．

一方，アゴニスティックな抗 Fas 抗体（Jo2 抗体）で誘導される致死的な急性肝不全モデルに組換えヒト HGF を単回投与すると，肝におけるアポトーシス細胞は減少し，生存率は著明に改善する（図2）．このような HGF の効果は抗アポトーシス作用によるものとされており，D-ガラクトサミン（D-GalN），リポ多糖（LPS），四塩化炭素などによる他の肝障害モデルでも確認され，その機序は Bcl-xL を介したものとされている[11~13]．

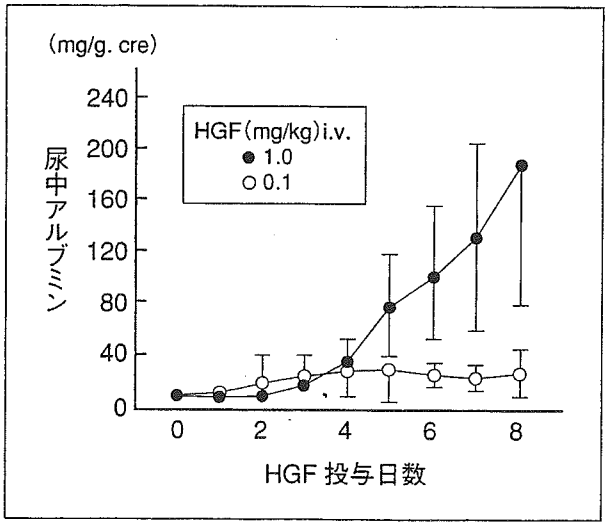
これらの急性肝障害モデルにおける HGF の効果から，劇症肝炎患者に対する組換えヒト HGF 投与が肝再生促進のみならず抗アポトーシス作用を介した病態進展阻止にも大き

図2 抗 Fas（Jo2）抗体誘導急性肝不全モデルにおける組換えヒト HGF の生存率改善効果




アゴニスティックな抗 Fas（Jo2）抗体（8μg）をマウス腹腔内に単回投与すると致死的な急性肝不全が誘導されるが，組換えヒト HGF 0.1mg を単回腹腔内投与すると生存率が著明に改善する．

図3 組換えヒト HGF 反復静脈内投与による尿中アルブミンの増加




ラットに組換えヒト HGF 1.0mg/kg を 8 日間反復静脈内投与すると，尿中アルブミンが増加する．尿中アルブミンの増加はタンパク尿に先行することから，尿中微量アルブミン測定は HGF による腎毒性のモニタリングマーカーとなりうることが考えられる．また，BUN やクレアチニン上昇といった腎機能障害を来す個体は見られなかった．

く作用することが期待される．特に予後不良な劇症肝炎亜急性型において，HGF の病態進展阻止効果は大きな意義を持つものと考えられる．

## 組換えヒト HGF の非臨床試験における問題点

### 1．薬理動態

組換えヒト HGF を静脈内投与するとその半減期は 2.4 分と短いが，静脈内投与でも肝臓における c-Met チロシンリン酸化が誘導され[14]，急性肝不全や肝硬変モデルに対する HGF の有効性は静脈内投与で確認されている．一方，静脈内投与された組換えヒト HGF の多くは肝臓に移行し，肝臓以外の臓器では副腎，脾臓，腎臓に移行する[14]．

### 2．安全性試験

これまで人体に投与されたことのない組換えヒト HGF の安全性をいかに確保するかが，その臨床応用における最も大きな課題である．我々は，「バイオテクノロジー応用医薬品の非臨床における安全性評価」ガイドラインに基づいて非臨床試験を進めている．

単回投与毒性試験では特に問題となる所見はなく，致死量は 40mg/kg 以上である．しかし，ラットを用いた反復投与毒性試験ではタンパク尿が出現し，腎組織では糸球体の増殖性変化が認められた．しかしこれらの腎毒性は可逆性で，HGF の休薬で軽減し，BUN やクレアチニンの上昇は見られない．さらに，組換えヒト HGF の反復投与では尿中微量アルブミンも増加するが（図3），この変化はタンパク尿に先行して出現することから，腎毒性のモニタリングマーカーとして有用であると考えている．また，組換えヒト HGF の呼吸器系および循環器系に及ぼす影響を解析する安全性薬理試験も必要と考えられるため，現在ミニブタを用いた試験を実施している．

肝細胞をはじめとした種々の上皮系細胞に対して増殖促進作用を持つ HGF の発癌性は，慎重に判断せねばならない．HGF のトランスジェニックマウスを用いた実験では，

表1　劇症肝炎および遅発性肝不全の救命率（1998～2002年）

| | 急性型 (n=272) | 亜急性型 (n=280) | 遅発性肝不全 (n=52) |
|---|---|---|---|
| 救命率（%）（例数） | | | |
| 内科的治療 | 54.0（128/237） | 23.4（48/205） | 12.2（5/41） |
| 肝移植 | 74.3（26/35） | 80.0（60/75） | 72.7（8/11） |
| 全体 | 56.6（154/272） | 38.6（108/280） | 25.0（13/52） |

（厚生労働省特定疾患対策研究事業「難治性の肝疾患に対する研究班」平成15年度報告書[18]より引用改変）

その発現量と発現臓器に差があるものの，発癌促進または抑制という相反する成績が報告されている[15~17]．我々は発癌性試験として，肝硬変を背景に肝癌を発生するコリン欠乏アミノ酸置換食飼育ラットに組換えヒトHGFを9ヵ月間反復投与し，その肝前癌病変および肝癌の発生に及ぼす影響を検討したが，HGF投与はむしろ発癌を抑制する傾向を示した（未発表データ）．しかし，どんなに発癌性試験を重ねても，増殖因子であるHGFの発癌性を完全に否定することはできない．したがって，HGFによる発癌性は否定できないというスタンスで，リスク&ベネフィットの観点から臨床試験の妥当性を検討する必要があると考えられた．

## 劇症肝炎および遅発性肝不全への臨床応用に向けて

組換えヒトHGFの臨床応用は，その発癌性のリスクから当初より劇症肝炎および遅発性肝不全を対象とした第Ⅰ・Ⅱ相とし，医師が主導する治験として実施する計画である．これまで人体に投与されたことのない組換えヒトHGFの臨床応用では，その臨床試験の妥当性が何より重要である．現在，劇症肝炎および遅発性肝不全において唯一肝移植のみが70～80%の救命率が期待される治療となっているが，内科的治療による救命率はいまだ低く（表1），肝移植以外に救命率を改善する有効な治療法が確立されていないのが現状である[18][19]．我々は急性肝不全研究会で提唱された肝移植ガイドライン（表2）に基づき[20]，肝移植が実施されねば予後不良すなわち死亡と推測される症例のうち，肝移植が実施できない症例を対象とすることを考えている．劇症肝炎の全国調査データによると肝移植ガイドラインで肝移植の適応とされた肝移植非実施例では約75%が死亡していること，また肝移植以外に有効な治療法が確立されていないことから，我々の選択規準は理解が得られるものと考えている．劇症肝炎および遅発性肝不全の中でもこのような重症例を対象として，果たしてHGFの有効性を評価できるのかは疑問が残るところであるが，初めて人体に投与される組換えヒトHGFの臨床試験では，その安全性（有害事象の有無）を評価することが何よりも優先される事項であり，HGFのような新規治療の開発はあくまで現時点における標準治療で効果の得られない症例に絞って行われるべきものであると考えている．もちろん患者を対象とした試験であるので，有効性のない試験を行うことにも倫理的問題があると考えられるため，動物モデルにおいて有効性が証明されている投与量から開始する計画である．

組換えヒトHGFの安全性試験では，腎毒性が問題となっている．我々はタンパク尿の可逆性について追加試験を行い，腎臓専門医

表2 劇症肝炎に対する肝移植のガイドライン（文献[20]より引用）

Ⅰ．脳症発現時に次の5項目のうち2項目を満たす場合は死亡と予測して肝移植の登録を行う
　1．年齢：45歳以上
　2．亜急性型（初発症状から脳症発現までの日数：11日以上）
　3．プロトロンビン時間：10%未満
　4．総ビリルビン濃度：18.0mg/dl以上
　5．直接/総ビリルビン比：0.67以下

Ⅱ．治療開始（脳症発現）から5日後における予後の再予測
　1．脳症がⅠ度以内に覚醒，あるいは昏睡度でⅡ度以上の改善
　2．プロトロンビン時間が50%以上に改善

以上のうちで，認められる項目数が
　2項目以上の場合：生存と予測して肝移植の登録を取り消す
　0または1項目の場合：死亡と再予測して肝移植の登録を継続する

の協力を得て腎病理所見が可逆性の変化にとどまることを確認し，糸球体障害が示唆される症例は対象から除外する方針である．一方，劇症肝炎および遅発性肝不全では約30%に腎障害が合併する．HGFによる腎障害と肝腎症候群との鑑別は困難となることが予測されるが，組換えヒトHGFの腎機能に及ぼす影響を詳細に解析する方針であるとともに，HGF投与中は尿中微量アルブミンなどで腎障害のモニタリングを行い，糸球体障害を示唆する所見が出現した際には早期にHGFの休薬または治療の中止とする方針である．

一方，増殖因子であるHGFが発癌を促進する可能性は，発癌性試験で発癌を促進するデータが得られなくてもゼロとは言えない．したがって，悪性腫瘍の既往がある症例を除外することはもとより，組換えヒトHGF投与によって悪性腫瘍の発生が促進される可能性は否定できないというスタンスで十分なインフォームド・コンセントを行うこと，また有効な治療法のない予後不良の劇症肝炎および遅発性肝不全を対象とするため，リスク&ベネフィットの観点から理解が得られるものと考えている．また，組換えヒトHGFが投与された救命例には数年間の定期検診を実施する計画である．

## おわりに

我々はHGFの精製以来，まずHGFの測定法を確立し，血清HGF測定が劇症肝炎の予後予測や劇症化の予知に有用であることを明らかにした．その後，製薬会社と共同でHGFの医薬品化を進め，ようやく組換えヒトHGFの臨床試験が実現可能となる時期が到来した．まずは肝移植以外に有効な治療法が確立されていない劇症肝炎および遅発性肝不全を対象に，組換えヒトHGFの臨床試験を始めたいと考えている．このようなHGFを用いたトランスレーショナルリサーチにおいては，安全性を確保することは何より優先される事項であるが，十二分に議論を尽くして倫理的に承認された臨床試験を実施すること，さらに客観的評価に耐えうる臨床試験のデータを得ることが大変重要である．また，これら臨床試験の実施にかかるプロセスの透明性も確保されるべき事項である．組換えヒトHGFの劇症肝炎および遅発性肝不全に対する臨床試験は2005年内には開始される見通しであるが，果たしてHGFが劇症肝炎に対する安全で有効な治療薬となるのか否か，

明確にしたいと考えている．

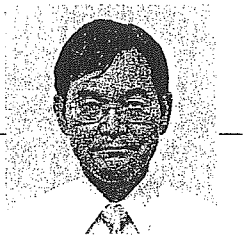
## 文　　献


1) Gohda E, et al: Human hepatocyte growth factor in patients with fulminant hepatic failure. Exp Cell Res 166: 139–150, 1986.
2) Gohda E, et al: Purification and partial characterization of hepatocyte growth factor from plasma of a patient with fulminant hepatic failure. J Clin Invest 81: 414–419, 1988.
3) 坪内博仁: 肝細胞増殖因子 (HGF). 肝臓 39: 413–427, 1998.
4) 合田榮一: 肝細胞増殖因子 (HGF) の機能と産生制御. 日薬理誌 119: 287–294, 2002.
5) Tsubouchi H, et al: Clinical significance of human hepatocyte growth factor in blood from patients with fulminant hepatic failure. Hepatology 9: 875–881, 1989.
6) Tsubouchi H, et al: Levels of the human hepatocyte growth factor in serum of patients with various liver diseases determined by an enzyme–linked immunosorbent assay. Hepatology 13: 1–5, 1991.
7) Tsubouchi H, et al: Prediction of outcome in fulminant hepatic failure by serum human hepatocyte growth factor. Lancet 340: 307, 1992.
8) Ljubimova J Y, et al: Lack of hepatocyte growth factor receptor (c–met) gene expression in fulminant hepatic failure livers before transplantation. Dig Dis Sci 42: 1675–1680, 1997.
9) Eguchi S, et al: Changes in liver regenerative factors in a case of living–related liver transplantation. Clin Transplant 13: 536–544, 1999.
10) Miwa Y, et al: Plasma levels and hepatic mRNA expression of transforming growth factor–$\beta$1 in patients with fulminant hepatic failure. J Hepatol 27: 780–788, 1997.
11) Kosai K, et al: Abrogation of Fas–induced fulminant hepatic failure in mice by hepatocyte growth factor. Biochem Biophys Res Commun 244: 683–690, 1998.
12) Masunaga H, et al: Preventive effects of the deleted form of hepatocyte growth factor against various liver injuries. Eur J Pharmacol 342: 267–279, 1998.
13) Kosai K, et al: Hepatocyte growth factor prevents endotoxin–induced lethal hepatic failure in mice. Hepatology 30: 151–159, 1999.
14) Ido A, et al: Pharmacokinetic study of recombinant human hepatocyte growth factor administered in a bolus intravenously or via portal vein. Hepatol Res 30: 175–181, 2004.
15) Santoni–Rugiu E, et al: Inhibition of neoplastic development in the liver by hepatocyte growth factor in a transgenic mouse model. Proc Natl Acad Sci USA 93: 9577–9582, 1996.
16) Sakata H, et al: Hepatocyte growth factor/scatter factor overexpression induces growth, abnormal development, and tumor formation in transgenic mouse livers. Cell Growth Differ 7: 1513–1523, 1996.
17) Takayama H, et al: Diverse tumorigenesis associated with aberrant development in mice overexpressing hepatocyte growth factor/scatter factor. Proc Natl Acad Sci USA 94: 701–706, 1997.
18) 藤原研司, 他: 劇症肝炎および遅発性肝不全 (LOHF: late onset hepatic failure) の全国集計 (2002 年). 厚生労働省特定疾患克服研究事業「難治性の肝疾患に関する調査研究」平成 15 年度研究報告書, p85–96, 2004.
19) 持田 智, 他: LOHF の現況 わが国における劇症肝炎, LOHF の実態. 日消病会誌 99: 895–904, 2002.
20) 杉原潤一, 他: わが国における劇症肝炎の予後予測と肝移植の適応に関する多施設研究. 肝臓 42: 543–557, 2001.

Prog.Med.
25：1611～1615, 2005

特集 生活習慣病と非アルコール性脂肪肝炎(NASH)


楠元 寿典


坪内 博仁

# 生活習慣病としてのNASH

Kusumoto Kazunori 楠元 寿典[1)]　Tsubouchi Hirohito 坪内 博仁[2)]

[1)]宮崎大学医学部第二内科 [2)]鹿児島大学大学院医歯学総合研究科消化器疾患・生活習慣病学教授

## はじめに

近年，日常診療の場で生活習慣病に関連した脂肪肝によく遭遇するが，その多くは非アルコール性脂肪性肝障害(non-alcoholic fatty liver disease；NAFLD)であると考えられる．NAFLDには肝硬変，肝癌へと進行する非アルコール性脂肪肝炎(non-alcoholic steatohepatitis；NASH)が含まれており，注意が必要である．これらはメタボリックシンドロームとの関連も指摘されており，それらの知見を併せて紹介する．

## 生活習慣病の定義

生活習慣病は，1996年に厚生省(現・厚生労働省)が提案した疾患概念である．それまでは主として，「脳卒中，がんなどの悪性腫瘍，心臓病などの40歳前後から急に死亡率が高くなり，しかも全死因の中でも高位を占め，40～60歳位の働き盛りに多い疾患群」に対して，「成人病」という行政用語が用いられていた．しかし近年，「成人病」の発症には生活習慣が深く関与していることが明らかになり，従来の「成人病」に加えて生活習慣が密接に関連する疾患を「生活習慣病」と呼ぶようになった．すなわち，生活習慣病は，脳卒中，癌，心臓病に加えて糖尿病，高脂血症，骨粗鬆症などを含む，「食習慣，運動習慣，休養，喫煙，飲酒等の生活習慣が，その発症・進行に関与する疾患群」と幅広く定義されている．

厚生労働省は，生活習慣病に対し，2010年を目指して平成13年から21世紀における国民健康づくり運動(健康日本21)を開始した．健康日本21では，9分野における生活習慣や生活習慣病について，対策と目標を示している．食生活・栄養の分野では，過剰摂取による健康障害を防ぐための上限値を初めて設定し，対応を開始した．身体活動・運動の分野でも，インスリン抵抗性を改善するために，運動，散歩および体重などの具体的な目標を掲げている[1,2]．

## 生活習慣病としてのNASH

### 1．検診における肝機能障害とNASHの頻度

2001年人間ドック全国集計成績によると，肝機能異常者の頻度は1984年には10%以下であったのに対して，最近では25%以上と増加している．これはアルコール性肝疾患をはじめ，肥満，糖尿病，高脂血症などの生活習慣病に関連するNAFLDおよびNASHが含まれていると考えられる[3]．本邦でも食生活の欧米化に伴い，この20年間に肥満人口は倍増し約2,300万人を数え，現在も肥満は若い女性を除いて増加傾向(図1)にある．これに伴い，現在日本におけるNAFLDの有病率は成人10人に1人程度，NASHは100人に1人弱程度と推計されており，今後生活習慣病の一肝病変であるNAFLD，NASHはますます増加すると考えられる[4]．

### 2．生活習慣病とNASHの概念

脂肪肝の原因は，大きくアルコール性と非アルコール性に分類(図2)される[5-7]が，このうち患者数が最も多く問題となるのが，生活習慣病を基盤とする

0287-3648/05/¥500/論文/JCLS