ACACA compensates for the increased accumulation of triglycerides.

Of the genes involved in lipid uptake, fatty acid transporter protein 5, a liver-specific member of the fatty acid transporter protein family, mediates the uptake of long-chain fatty acids. Unexpectedly, the expression of *SLC27A5* (encoding fatty acid transporter protein 5) was not up-regulated but rather down-regulated in patients with steatosis. Again, this expression could be a compensatory response to increased accumulation of triglyceride.

Further studies are needed to determine the importance of the products of these genes because the limited size of biopsy samples prevented measurement of the enzyme activities. Changes in enzymatic activities of their products are more important for the development of steatosis than changes in their transcriptional levels. Moreover, in vitro studies and mouse models have shown that HCV proteins cause mitochondrial injury, leading to oxidative stress [39–43]. Oxidative stress may inhibit enzymes involved in lipid metabolism, and reactive oxygen species may cause peroxidation of membrane lipids and structural proteins, such as those involved in trafficking and secretion of lipids. Oxidative stress perturbs lipid metabolism, thus contributing to steatosis. It is possible that, instead of a direct effect of HCV proteins on the transcription of genes regulating lipid metabolism, nonspecific inhibition of lipid metabolism through oxidative stress leads to HCV-related steatosis.

In conclusion, a higher BMI, higher levels of γ-GTP and triglyceride, and a higher fibrosis stage correlate independently with steatosis in HCV-infected Japanese patients. Thus, the down-regulation of genes involved in fatty acid oxidation may contribute to the development of steatosis in these patients.

Acknowledgments This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Program of Science (20590408) and by a grant from Ministry of Health, Labor and Welfare of Japan (H20-hepatitis-008; to K. Yasui).

References

1. Asselah T, Bieche I, Narguet S, Sabbagh A, Laurendeau I, Ripault MP, et al. Liver gene expression signature to predict response to pegylated interferon plus ribavirin combination therapy in patients with chronic hepatitis C. Gut. 2008;57:516–24.
2. Clark JM, Brancati FL, Diehl AM. Nonalcoholic fatty liver disease. Gastroenterology. 2002;122:1649–57.
3. Negro F. Mechanisms and significance of liver steatosis in hepatitis C virus infection. World J Gastroenterol. 2006;12:6756–65.
4. Poynard T, McHutchison J, Manns M, Myers RP, Albrecht J. Biochemical surrogate markers of liver fibrosis and activity in a randomized trial of peginterferon alfa-2b and ribavirin. Hepatology. 2003;38:481–92.
5. Leandro G, Mangia A, Hui J, Fabris P, Rubbia-Brandt L, Colloredo G, et al. Relationship between steatosis, inflammation, and fibrosis in chronic hepatitis C: a meta-analysis of individual patient data. Gastroenterology. 2006;130:1636–42.
6. Mihm S, Fayyazi A, Hartmann H, Ramadori G. Analysis of histopathological manifestations of chronic hepatitis C virus infection with respect to virus genotype. Hepatology. 1997;25:735–9.
7. Rubbia-Brandt L, Quadri R, Abid K, Giostra E, Malé PJ, Mentha G, et al. Hepatocyte steatosis is a cytopathic effect of hepatitis C virus genotype 3. J Hepatol. 2000;33:106–15.
8. Kumar D, Farrell GC, Fung C, George J. Hepatitis C virus genotype 3 is cytopathic to hepatocytes: reversal of hepatic steatosis after sustained therapeutic response. Hepatology. 2002;36:1266–72.
9. Monto A, Alonzo J, Watson JJ, Grunfeld C, Wright TL. Steatosis in chronic hepatitis C: relative contributions of obesity, diabetes mellitus, and alcohol. Hepatology. 2002;36:729–36.
10. Barba G, Harper F, Harada T, Kohara M, Goulinet S, Matsuura Y, et al. Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. Proc Natl Acad Sci USA. 1997;94:1200–5.
11. Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, Matsuura Y, et al. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. J Gen Virol. 1997;78:1527–31.
12. Perlemuter G, Sabile A, Letteron P, Vona G, Topilco A, Chrétien Y, et al. Hepatitis C virus core protein inhibits microsomal triglyceride transfer protein activity and very low density lipoprotein secretion: a model of viral-related steatosis. FASEB J. 2002;16:185–94.
13. Mirandola S, Realdon S, Iqbal J, Gerotto M, Dal Pero F, Bortoletto G, et al. Liver microsomal triglyceride transfer protein is involved in hepatitis C liver steatosis. Gastroenterology. 2006;130:1661–9.
14. Su AI, Pezacki JP, Wodicka L, Brideau AD, Supekova L, Thimme R, et al. Genomic analysis of the host response to hepatitis C virus infection. Proc Natl Acad Sci USA. 2002;99:15669–74.
15. Kim KH, Hong SP, Kim K, Park MJ, Kim KJ, Cheong J. HCV core protein induces hepatic lipid accumulation by activating SREBP1 and PPARgamma. Biochem Biophys Res Commun. 2007;355:883–8.
16. Tsutsumi T, Suzuki T, Shimoike T, Suzuki R, Moriya K, Shintani Y, et al. Interaction of hepatitis C virus core protein with retinoid X receptor alpha modulates its transcriptional activity. Hepatology. 2002;35:937–46.
17. Yamaguchi A, Tazuma S, Nishioka T, Ohishi W, Hyogo H, Nomura S, et al. Hepatitis C virus core protein modulates fatty acid metabolism and thereby causes lipid accumulation in the liver. Dig Dis Sci. 2005;50:1361–71.
18. Cheng Y, Dharancy S, Malapel M, Desreumaux P. Hepatitis C virus infection down-regulates the expression of peroxisome proliferator-activated receptor alpha and carnitine palmitoyl acyl-CoA transferase 1A. World J Gastroenterol. 2005;11:7591–6.
19. Dharancy S, Malapel M, Perlemuter G, Roskams T, Cheng Y, Dubuquoy L, et al. Impaired expression of the peroxisome proliferator-activated receptor alpha during hepatitis C virus infection. Gastroenterology. 2005;128:334–42.
20. Japan Society for the Study of Obesity. New criteria of obesity (in Japanese). J Jpn Soc Study Obes. 2000;6:18–28.
21. Simmonds P, Alberti A, Alter HJ, Bonino F, Bradley DW, Brechot C, et al. A proposed system for the nomenclature of hepatitis C viral genotypes. Hepatology. 1994;19:1321–4.
22. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology. 1994;19:1513–20.

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. Lefkowitch JH, Schiff ER, Davis GL, Perrillo RP, Lindsay K, Bodenheimer HC Jr, et al. Pathological diagnosis of chronic hepatitis C: a multicenter comparative study with chronic hepatitis B. The Hepatitis Interventional Therapy Group. Gastroenterology. 1993;104:595–603.
25. Lonardo A, Loria P, Adinolfi LE, Carulli N, Ruggiero G. Hepatitis C and steatosis: a reappraisal. J Viral Hepat. 2006;13:73–80.
26. Akuta N, Suzuki F, Tsubota A, Suzuki Y, Someya T, Kobayashi M, et al. Efficacy of interferon monotherapy to 394 consecutive naive cases infected with hepatitis C virus genotype 2a in Japan: therapy efficacy as consequence of tripartite interaction of viral, host and interferon treatment-related factors. J Hepatol. 2002;37:831–6.
27. Ohata K, Hamasaki K, Toriyama K, Matsumoto K, Saeki A, Yanagi K, et al. Hepatic steatosis is a risk factor for hepatocellular carcinoma in patients with chronic hepatitis C virus infection. Cancer. 2003;97:3036–43.
28. Fujie H, Yotsuyanagi H, Moriya K, Shintani Y, Tsutsumi T, Takayama T, et al. Steatosis and intrahepatic hepatitis C virus in chronic hepatitis. J Med Virol. 1999;59:141–5.
29. Castera L, Chouteau P, Hezode C, Zafrani ES, Dhumeaux D, Pawlotsky JM. Hepatitis C virus-induced hepatocellular steatosis. Am J Gastroenterol. 2005;100:711–5.
30. Westin J, Nordlinder H, Lagging M, Norkrans G, Wejstål R. Steatosis accelerates fibrosis development over time in hepatitis C virus genotype 3 infected patients. J Hepatol. 2002;37:837–42.
31. Patton HM, Patel K, Behling C, Bylund D, Blatt LM, Vallée M, et al. The impact of steatosis on disease progression and early and sustained treatment response in chronic hepatitis C patients. J Hepatol. 2004;40:484–90.
32. Fartoux L, Chazouillères O, Wendum D, Poupon R, Serfaty L. Impact of steatosis on progression of fibrosis in patients with mild hepatitis C. Hepatology. 2005;41:82–7.
33. Adinolfi LE, Gambardella M, Andreana A, Tripodi MF, Utili R, Ruggiero G. Steatosis accelerates the progression of liver damage of chronic hepatitis C patients and correlates with specific HCV genotype and visceral obesity. Hepatology. 2001;33:1358–64.
34. Ikai E, Honda R, Yamada Y. Serum gamma-glutamyl transpeptidase level and blood pressure in nondrinkers: a possible pathogenetic role of fatty liver in obesity-related hypertension. J Hum Hypertens. 1994;8:95–100.
35. Lee DS, Evans JC, Robins SJ, Wilson PW, Albano I, Fox CS, et al. Gamma glutamyl transferase and metabolic syndrome, cardiovascular disease, and mortality risk: the Framingham Heart Study. Arterioscler Thromb Vasc Biol. 2007;27:127–33.
36. Perry IJ, Wannamethee SG, Shaper AG. Prospective study of serum gamma-glutamyltransferase and risk of NIDDM. Diabetes Care. 1998;21:732–7.
37. de Gottardi A, Pazienza V, Pugnale P, Bruttin F, Rubbia-Brandt L, Juge-Aubry CE, et al. Peroxisome proliferator-activated receptor-alpha and -gamma mRNA levels are reduced in chronic hepatitis C with steatosis and genotype 3 infection. Aliment Pharmacol Ther. 2006;23:107–14.
38. Desvergne B, Wahli W. Peroxisome proliferator-activated receptors: nuclear control of metabolism. Endocr Rev. 1999;20:649–88.
39. Moriya K, Nakagawa K, Santa T, Shintani Y, Fujie H, Miyoshi H, et al. Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. Cancer Res. 2001;61:4365–70.
40. Okuda M, Li K, Beard MR, Showalter LA, Scholle F, Lemon SM, et al. Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. Gastroenterology. 2002;122:366–75.
41. Lerat H, Honda M, Beard MR, Loesch K, Sun J, Yang Y, et al. Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. Gastroenterology. 2002;122:352–65.
42. Lai MM. Hepatitis C virus proteins: direct link to hepatic oxidative stress, steatosis, carcinogenesis and more. Gastroenterology. 2002;122:568–71.
43. Asselah T, Rubbia-Brandt L, Marcellin P, Negro F. Steatosis in chronic hepatitis C: why does it really matter? Gut. 2006; 55:123–30.

ELSEVIER

Cancer Genetics and Cytogenetics 198 (2010) 118–125

CANCER GENETICS AND CYTOGENETICS

PEG10 is a probable target for the amplification at 7q21 detected in hepatocellular carcinoma

Kazuhiro Tsuji[a], Kohichiroh Yasui[a,*], Yasuyuki Gen[a], Mio Endo[a], Osamu Dohi[a], Keika Zen[a], Hironori Mitsuyoshi[a], Masahito Minami[a], Yoshito Itoh[a], Masafumi Taniwaki[b], Shinji Tanaka[c], Shigeki Arii[c], Takeshi Okanoue[a,d], Toshikazu Yoshikawa[a]

[a]*Department of Molecular Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kamigyo-ku, Kyoto 602-8566, Japan*

[b]*Department of Molecular Hematology and Oncology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan*

[c]*Department of Hepato-Biliary-Pancreatic Surgery, Tokyo Medical and Dental University, Tokyo, Japan*

[d]*Center of Gastroenterology and Hepatology, Saiseikai Suita Hospital, Suita, Osaka, Japan*

Received 3 September 2009; received in revised form 3 January 2010; accepted 3 January 2010

Abstract DNA copy number aberrations in human hepatocellular carcinoma (HCC) cell lines were investigated using a high-density oligonucleotide microarray, and a novel amplification at the chromosomal region 7q21 was detected. Molecular definition of the amplicon indicated that *PEG10* (paternally expressed gene 10), a paternally expressed imprinted gene, was amplified together with *CDK14* (cyclin-dependent kinase 14; previously PFTAIRE protein kinase 1, *PFTK1*) and *CDK6* (cyclin-dependent kinase 6). An increase in *PEG10* copy number was detected in 14 of 34 primary HCC tumors (41%). *PEG10*, but not *CDK14* or *CDK6*, was significantly overexpressed in 30 of 41 tumors (73%) from HCC patients, compared with their nontumorous counterparts. These results suggest that *PEG10* is a probable target, acting as a driving force for amplification of the 7q21 region, and may therefore be involved in the development or progression of HCCs. © 2010 Elsevier Inc. All rights reserved.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in men and the eighth most common in women worldwide; it is estimated to cause approximately half a million deaths annually [1]. Although the risk factors for HCC, which include hepatitis B virus, hepatitis C virus, and alpha-toxin, are well characterized, the molecular pathogenesis of this widespread type of cancer remains poorly understood [2].

Amplification of DNA in certain regions of chromosomes plays a crucial role in the development and progression of human malignancies, specifically when protooncogenic target genes within those amplicons are overexpressed. Oncogenes that are often amplified in cancers include *MYC*, *ERBB2*, and *CCND1*. The recent introduction of high-density oligonucleotide microarrays designed for typing of single nucleotide polymorphisms (SNPs) facilitates high-resolution mapping of chromosomal amplifications, deletions, and losses of heterozygosity [3,4].

To identify genes potentially involved in HCC, we investigated DNA copy number aberrations in human HCC cell lines using high-resolution SNP arrays and found a novel amplification at the chromosomal region 7q21. Recurrent amplifications at 7q21 have been observed in human neoplasms [5]. Gains of 7q21 have been associated with the aggressiveness of several tumors, including HCC [6], colorectal cancer [7], prostate cancer [8], Burkitt lymphoma [9], and esophageal squamous cell carcinoma [10]. These data suggest that this chromosomal region may harbor one or more protooncogenes (henceforth referred to as *target genes*) whose overexpression following amplification might contribute to the initiation or progression of HCC. The actual target gene that drives the 7q21 amplification in HCC remains unclear, however, and we therefore conducted a molecular definition study of the amplicon to identify such genes. Three putative oncogenes, *CDK14* (cyclin-dependent kinase 14; previously *PFTK1*, PFTAIRE protein kinase 1), *CDK6* (cyclin-dependent

* Corresponding author. Tel.: +81-75-251-5519; fax: +81-75-251-0710.

E-mail address: yasuik@koto.kpu-m.ac.jp (K. Yasui).

0165-4608/10/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.cancergencyto.2010.01.004

kinase 6), and *PEG10* (paternally expressed gene 10), were identified in the 7q21 amplicon.

The serine/threonine-protein kinase PFTAIRE-1 protein (also known as PFTK1) is a member of the cell division cycle-2 (CDC2)-related protein kinase family [11] and acts as a cyclin-dependent kinase that regulates cell cycle progression and cell proliferation [12]. CDK6 is activated in response to increased expression of D-type cyclins in the early G1 phase of the cell cycle and inactivates the retinoblastoma protein by phosphorylation, thereby activating the transcriptional complex E2F-DP1 that regulates the genes for S-phase onset [13]. *PEG10* has been characterized as a paternally expressed, maternally silenced gene [14]. Several research groups have recently reported overexpression of *PEG10* in HCC [15–19].

2. Materials and methods

2.1. Cell lines and tumor samples

A total of 20 HCC cell lines were examined: JHH-1, JHH-2, JHH-4, JHH-5, JHH-6, JHH-7, SNU354, SNU368, SNU387, SNU398, SNU423, SNU449, SNU475, Huh-1, Huh7, Hep3B, PLC/PRF/5, Li7, HLE, and HLF [20]. All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Paired tumor and nontumor tissues were obtained from 36 HCC patients who underwent surgery at the Hospital of Tokyo Medical and Dental University. All specimens were frozen immediately in liquid nitrogen and were stored at −80°C until required. Genomic DNA was isolated using a Puregene DNA isolation kit (Gentra, Minneapolis, MN), and total RNA was obtained using Trizol reagent (Invitrogen, Carlsbad, CA). Thirty-four tumor samples were available for DNA analyses, and 41 paired tumor and nontumor samples were available for mRNA analyses.

Prior to the study, informed consent was obtained and the study was approved by ethics committees.

2.2. SNP array analysis

DNA copy number changes were analyzed by the GeneChip Mapping 100K array set (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions as described previously [21]. In brief, 250 ng of genomic DNA was digested with a restriction enzyme (*Xba*I or *Hin*dIII), ligated to an adaptor and amplified by polymerase chain reaction (PCR). Amplified products were fragmented, labeled by biotinylation, and hybridized to the microarrays. Hybridization was detected by incubation with a streptavidin–phycoerythrin conjugate, followed by scanning of the array; analysis was performed as previously described [22]. After appropriate normalization of mean array intensities, signal ratios were calculated between HCC cell lines and anonymous normal references, and copy numbers were inferred from the observed signal ratios based on the hidden Markov model using CNAG software (Copy Number Analyzer for Affymetrix GeneChip mapping arrays) [23]. The CNAG software is available at http://www.genome.umin.jp.

2.3. Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) was performed using five bacterial artificial chromosomes (BACs) as probes, as described previously [24]: RP11-66P5, RP11-412F4, RP11-316P4, RP11-28O23, and RP11-958G24 (Invitrogen, Carlsbad, CA). The BACs were selected based on their homology to locations in the human genome according to the database provided at the University of California, Santa Cruz, Genome Bioinformatics Web site (http://genome.ucsc.edu/).

2.4. Real-time quantitative PCR

Genomic DNA and mRNA were quantified using a real-time fluorescence detection method, as described previously [21]. The primers used for PCR (Table 1) were designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) on the basis of sequence data obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) database. *GAPDH* was used as endogenous control for mRNA levels, and the long interspersed nuclear element 1 (LINE-1) was used as an endogenous control for genomic DNA levels.

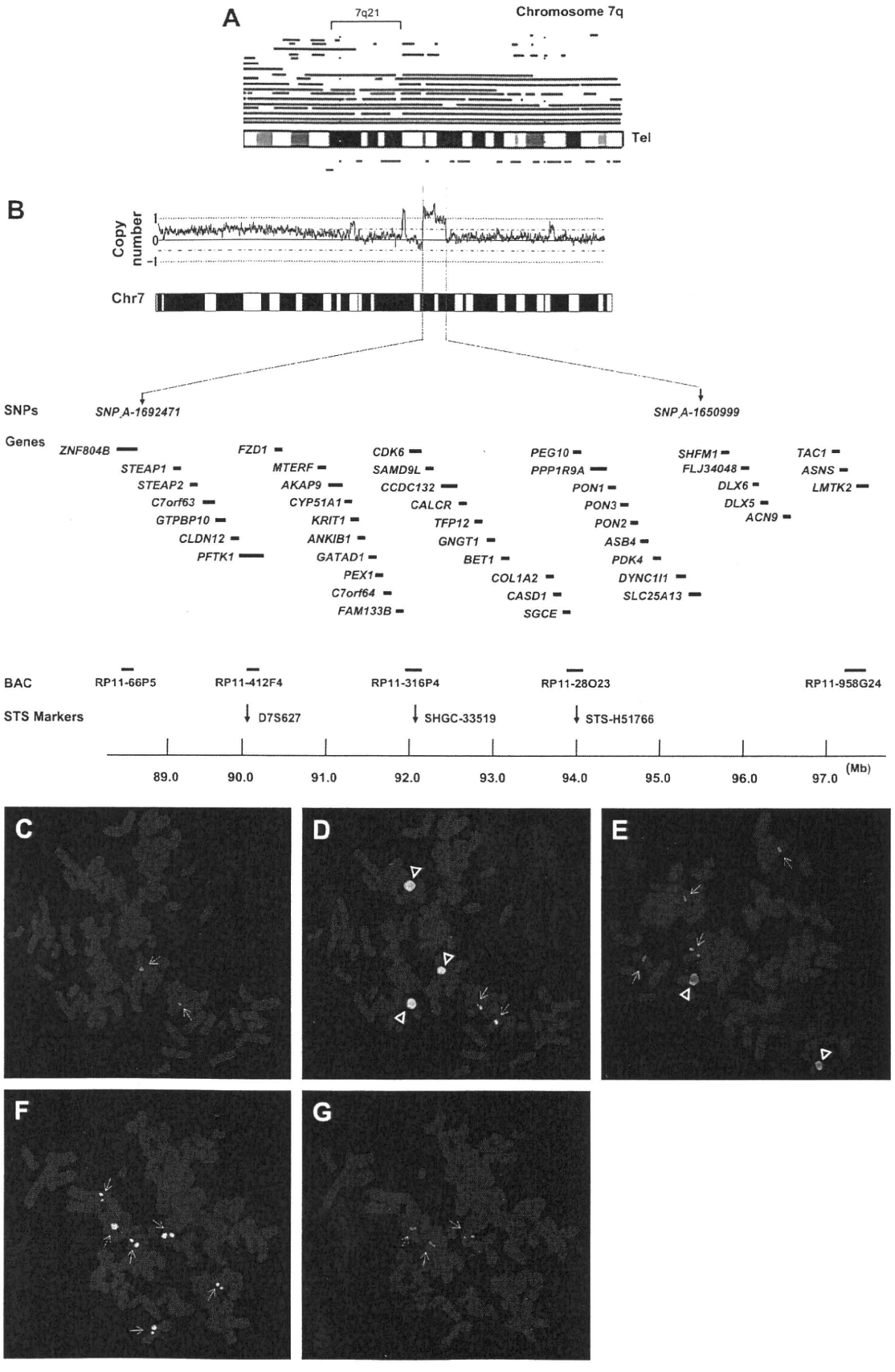
2.5. Statistical analysis

The Wilcoxon signed-rank test was performed using SPSS 15.0 software (SPSS, Chicago, IL). P values of <0.05 were considered significant.

Table 1
Primer sequences used for polymerase chain reaction with sequence-tagged site (STS) markers for the three genes investigated

Gene	STS marker	Forward primer	Reverse primer
CDK14 genomic DNA	D7S627	5′-AAACCAAGAACATTCCAG-3′	5′-ACACATCACATTCTCACC-3′
CDK14 mRNA		5′-CCAAGGAGTTGCTGCTTTTC-3′	5′-GAATGAACTCCAGGCCATGT-3′
CDK6 genomic DNA	SHGC-33519	5′-AAGTCAGAAGGAAAAAAGCTTACTG-3′	5′-TGAGATGTGTTAAAGTAGGTTTTCA-3′
CDK6 mRNA		5′AGCCCAAGATGACCAACATC-3′	5′-AGGTCAAGTTGGGAGTGGTG-3′
PEG10 genomic DNA	STS-H51766	5′-AAAGTTTACATACATTTATGAAGGG-3′	5′- TTCCAGACTGCACCATATAG-3′
PEG10 mRNA		5′-CAGGCCTGAAAAGAAAGTGC-3′	5′-AATGCTTTGTGGAAGCCATC-3′

The gene *CDK14* was previously assigned the symbol *PFTK1* (http://www.genenames.org).


A
7q21
Chromosome 7q
Tel
B
Copy number
1
0
-1
Chr7
SNPs
SNP_A-1692471
SNP_A-1650999
Genes
ZNF804B
STEAP1
STEAP2
C7orf63
GTPBP10
CLDN12
PFTK1
FZD1
MTERF
AKAP9
CYP51A1
KRIT1
ANKIB1
GATAD1
PEX1
C7orf64
FAM133B
CDK6
SAMD9L
CCDC132
CALCR
TFP12
GNGT1
BET1
COL1A2
CASD1
SGCE
PEG10
PPP1R9A
PON1
PON3
PON2
ASB4
PDK4
DYNC1I1
SLC25A13
SHFM1
FLJ34048
DLX6
DLX5
ACN9
TAC1
ASNS
LMTK2
BAC
RP11-66P5
RP11-412F4
RP11-316P4
RP11-28O23
RP11-958G24
STS Markers
D7S627
SHGC-33519
STS-H51766
89.0
90.0
91.0
92.0
93.0
94.0
95.0
96.0
97.0
(Mb)
C
D
E
F
G

3. Results

3.1. Detection of the 7q21 amplicon

Twenty HCC cell lines were screened for DNA copy number aberrations by GeneChip Mapping 100K array analysis. The copy number detection algorithm CNAG allowed assessment of copy number and identification of genomic gains and deletions using the hidden Markov model [23]. Gains at the chromosomal region 7q21 were frequently found in 13 of the 20 cell lines (65%) (Fig. 1A). Of these cell lines, JHH-4 cells exhibited a high-level copy number gain indicative of gene amplification at 7q21 (Fig. 1B). The estimated extent of the amplification in JHH-4 cells is 9 Mb. This chromosomal region lies between the Affymetrix markers SNP_A-1692471 and SNP_A-1650999 (supplementary Table S1) and includes 35 known or predicted protein-coding genes. The 7q21 region may harbor one or more genes that, when activated by amplification, play a role in carcinogenesis. Because we identified three putative oncogenes (i.e., *CDK14*, *CDK6*, and *PEG10*) in the 7q21 amplicon, we chose to focus further analysis on these three genes.

To confirm amplification of *CDK14*, *CDK6*, and *PEG10*, we performed FISH analyses on JHH-4 cells using the BACs RP11-66p5, RP11-412F4, RP11-316P4, RP11-28O23, and RP11-958G24 as probes. RP11-412F4 (containing *CDK14*) (Fig. 1D) and RP11-316P4 (containing *CDK6*) (Fig. 1E) generated amplified FISH signals, and RP11-28O23 (containing *PEG10*) (Fig. 1F) showed an increase in the number of FISH signals. In contrast, neither RP11-66P5 nor RP11-958G24, which correspond to chromosomal regions outside of the amplicon, showed an amplified signal or an increase in the number of FISH signals (Fig. 1C, 1G). These data confirm that *CDK14*, *CDK6*, and *PEG10* are amplified in JHH-4 cells.

3.2. DNA copy number and expression level of CDK14, CDK6, and PEG10 in HCC cell lines

To further analyze the potential role of *CDK14*, *CDK6*, and *PEG10* in HCC, we determined the DNA copy number of these three genes in 20 HCC cell lines by real-time quantitative PCR. For this analysis, copy number changes were counted as gains if the copy number for a given tumor cell type exceeded the mean plus 2 standard deviations of the level of the gene in normal cells. A copy number gain of *CDK14*, *CDK6*, and *PEG10* was observed in 13 (65%), 12 (60%), and 14 (70%) of the 20 cell lines, respectively (Fig. 2A). JHH-4 cells showed the highest copy number gain of each gene.

A common criterion for designation of a gene as a putative target of amplification is that gene amplification leads to its overexpression [25]. To determine whether *CDK14*, *CDK6*, and *PEG10* are overexpressed, we determined the mRNA level of these three genes in the 20 HCC cell lines by real-time quantitative PCR. Both *CDK6* and *PEG10*, but not *CDK14*, were overexpressed in JHH-4 cells, relative to the other cell lines (Fig. 2B). These findings suggested that *CDK6* and *PEG10* are candidate targets for the 7q21 amplification.

3.3. DNA copy number and expression level of CDK14, CDK6, and PEG10 in primary HCC tumors

To determine whether the amplification of *CDK14*, *CDK6*, and *PEG10* that was observed in JHH-4 cells was relevant to primary human carcinomas, we first determined the copy number of the three genes in 34 primary HCCs, using a method similar to that used for the HCC cell lines. A copy number gain of *CDK14*, *CDK6*, and *PEG10* was observed in 8 (24%), 16 (47%), and 14 (41%), respectively, of the 34 tumors (Fig. 3).

We then further examined the expression of the three genes in paired tumor and nontumor tissues from the 41 HCC patients by real-time quantitative PCR. Patient and tumor characteristics are summarized in Table 2. *PEG10* was significantly overexpressed in 30 of the 41 tumors (73%), compared with their nontumorous counterparts (Wilcoxon signed-rank test, $P < 0.001$) (Fig. 4). In contrast, expression of *CDK14* or *CDK6* was not upregulated in HCC tumors (Fig. 4). Taken together, these results suggest that *PEG10* is the most likely target for the 7q21 amplicon in HCC.

Fig. 1. Map of the amplicon at 7q21 in the human hepatocellular carcinoma (HCC) cell line JHH-4. (A) Recurrent copy number gains on the 7q arm as assessed using a GeneChip mapping 100K array (Affymetrix, Santa Clara, CA). Copy number gains are indicated by red horizontal lines above the chromosome ideogram: high-level gains (amplifications) are shown by bright red lines, whereas simple gains are shown by dark red lines. Copy number losses are indicated by green lines under the chromosome ideogram. Each horizontal line represents an aberration detected in a single HCC cell line. The cytobands in 7q are shown. (B) Copy number profile of chromosome 7 in JHH-4 cells. Copy number values were determined by GeneChip mapping 100K array analysis. Shown are the position of the Affymetrix single-nucleotide polymorphism (SNP) probes, the 35 genes included within the amplicon, the five bacterial artificial chromosomes (BACs) used as probes for fluorescence in situ hybridization (FISH) experiments, and the three sequence-tagged site (STS) markers used for real-time quantitative polymerase chain reaction (PCR) based on the University of California, Santa Cruz, Genome Bioinformatics database (http://genome.ucsc.edu/). (C-G) Representative images of FISH on metaphase chromosomes from JHH-4 cells, using the following BAC probes: paired RP11-66P5, containing *ZNF804B* (red) (C) and RP11-412F4, containing *CDK14* (green) (D); single RP11-316P4, containing *CDK6* (red) (E); and paired RP11-28O23, containing *PEG10* (green) (F) and RP11-958G24, containing *LMTK2* (red) (G). Arrows indicate normal signals; arrowheads indicate amplified signals. The set of images shows two normal signals (C), three amplified signals plus two normal signals (D), two amplified signals plus four normal signals (E), six normal signals (F), and three normal signals (G). *Note:* In these figures the gene *CDK14* is identified by the previously approved symbol, *PFTK1*.

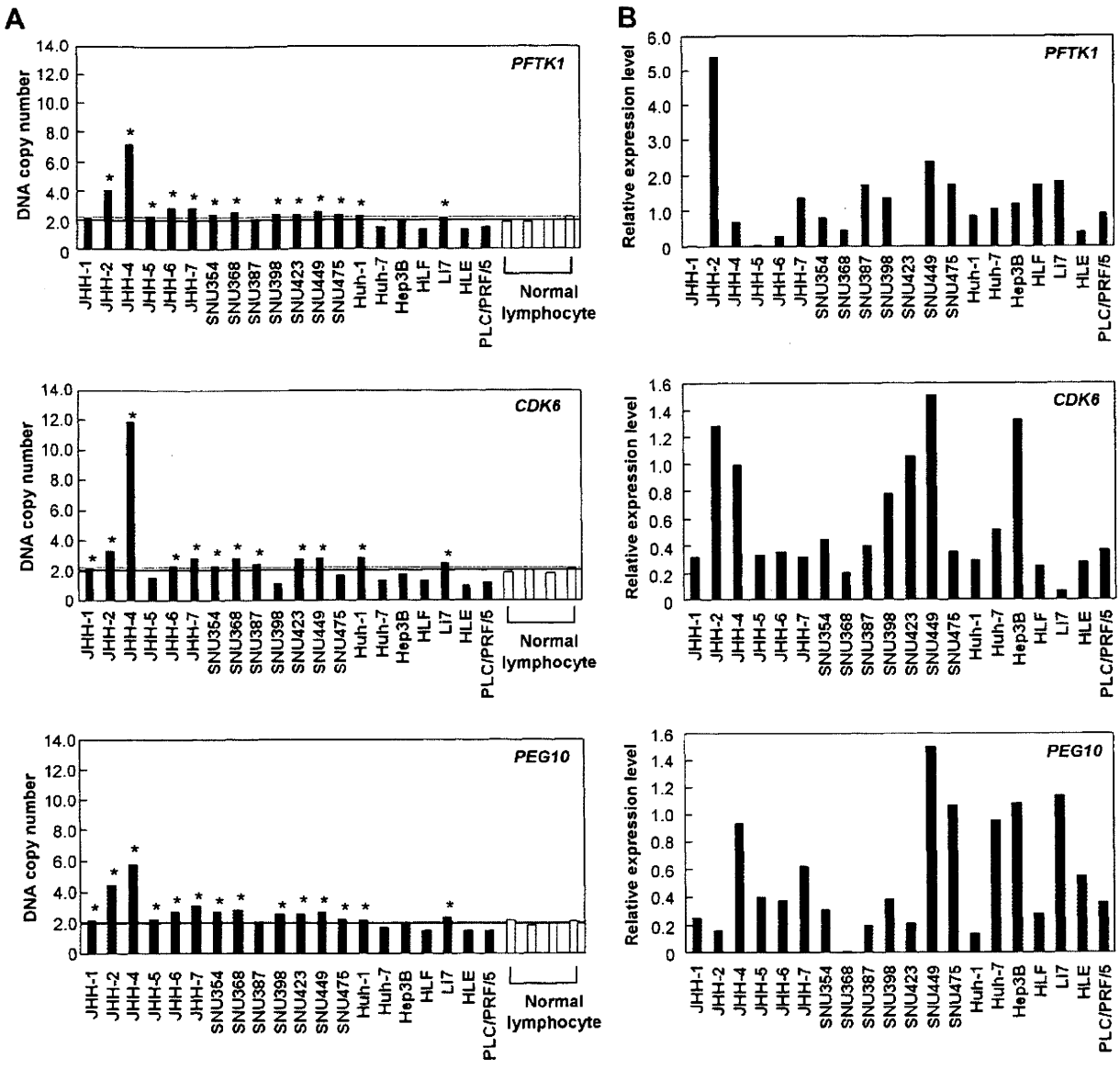


Fig. 2. DNA copy number and expression level of *CDK14* (previously *PFTK1*), *CDK6*, and *PEG10* in HCC cell lines. (A) DNA copy number of *CDK14*, *CDK6*, and *PEG10* in 20 HCC cell lines and four normal peripheral blood lymphocytes as measured by real-time quantitative PCR with reference to LINE-1 controls. Values are normalized such that the average copy number in genomic DNA derived from four normal lymphocytes has a value of 2 (solid horizontal line). A value corresponding to the mean +2 S.D. of the copy number of normal lymphocytes was used as the cutoff value for copy number gain (dotted line). Asterisks indicate cell lines showing copy number gain. (B) Relative expression levels of *CDK14*, *CDK6*, and *PEG10* in 20 HCC cell lines as determined by real-time quantitative PCR. The results are presented as the expression level of each gene relative to a reference gene (*GAPDH*), to correct for variations in the amount of RNA.

4. Discussion

The high-resolution SNP array analysis reported in this study identified amplification at the chromosomal region 7q21 in JHH-4 HCC cells. A copy number gain at this region was frequently observed, not only in HCC cell lines, but also in primary HCCs. Of the three genes identified in the amplicon (i.e., *PEG10*, *CDK6*, and *CDK14*), subsequent experiments suggested that *PEG10* is the most likely target for the amplicon, in that the *PEG10* transcript was both overexpressed in JHH-4 cells and significantly upregulated in primary HCC tumors, compared with their nontumorous counterparts. In contrast, although the highest level of copy number gain was found at the *CDK6* locus in JHH-4 cells and primary HCC tumors, *CDK6* expression was not upregulated in primary HCC tumors.

Contrary to these data, a recent report indicated that expression of *CDK14* was higher in HCC tumors than in

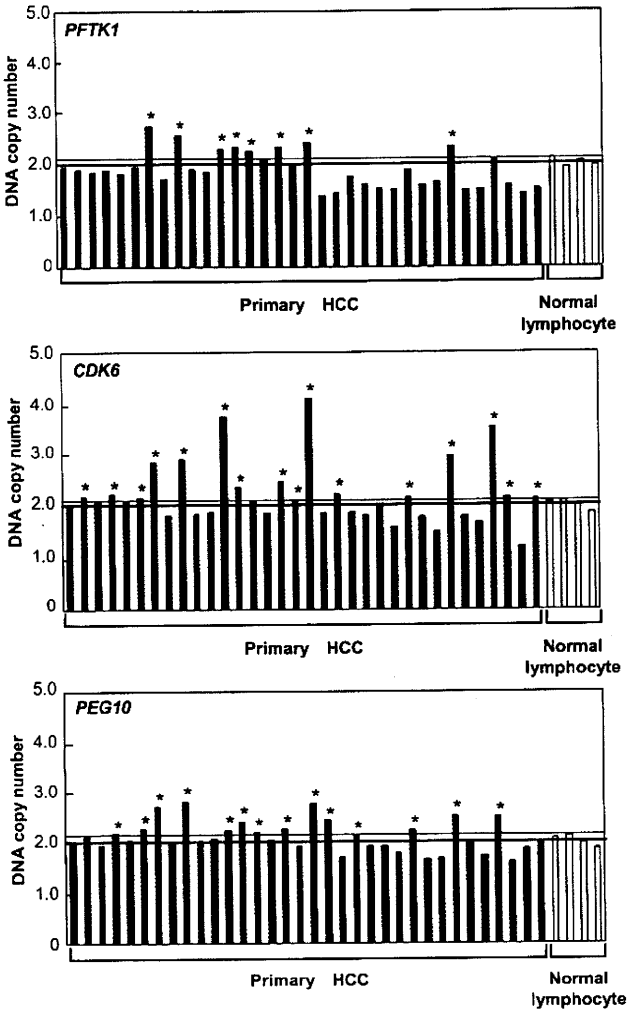


Fig. 3. DNA copy number of *CDK14* (previously *PFTK1*), *CDK6*, and *PEG10* in primary HCC tumors. The DNA copy number of each gene in 34 primary HCC tumors and four normal peripheral blood lymphocytes was determined as already described (for Fig. 2A). Asterisks indicate primary tumors showing copy number gain.

Table 2
Patient and tumor characteristics

Characteristics	Value[a]
Sample size[b]	n = 41
Sex	
Male	33
Female	8
Median age, yr (range)	67 (35–79)
Etiology of liver disease	
Hepatitis B virus	9
Hepatitis C virus	21
Other	11
Median tumor size, cm (range)	5.0 (1.9–26)
Tumors, single or multiple	
Single	26
Multiple	15
Tumor differentiation	
Well	7
Moderate	20
Poor	14
Stage[c]	
I	1
II	15
III	14
IV	11
Background liver tissue	
Normal	4
Chronic hepatitis	18
Liver cirrhosis	19
Child–Pugh classification	
A	40
B	1
C	0
Median α-fetoprotein, ng/mL (range)	14.9 (0.9–114,859)

[a] Where no other unit is specified, values refer to number of patients.
[b] All patients were of Japanese ethnicity.
[c] International Union Against Cancer tumor–node–metastasis (UICC TNM) classification of malignant tumor.

adjacent nontumorous liver tissues and that upregulation of the gene correlated with both advanced metastatic HCCs and microvascular invasion [26]. Further studies are required to clarify the potential role of *CDK14* in HCC.

PEG10 was first identified as an imprinted gene that is paternally expressed and maternally silenced [14]. It has been suggested that *PEG10* is derived from a retrotransposon that was previously integrated into the mammalian genome [14]. The overexpression of *PEG10* in HCC observed in this study is consistent with the previously reported overexpression of *PEG10* in tumors, including HCC [15–19], and in B-cell leukemia [27,28]. Furthermore, several lines of evidence suggest that *PEG10* may be important for the regulation of cell proliferation and cell death: *PEG10* is overexpressed in regenerating mouse liver [16], knockdown of *PEG10* inhibits the proliferation of cancer cells [29], and the PEG10 protein inhibits cell death mediated by SIAH1, a mediator of apoptosis [15]. The importance of *PEG10* for cell regulation is further suggested by the fact that targeted disruption of the mouse *Peg10* gene results in early embryonic lethality due to defects in the placenta [30].

The exact mechanism by which *PEG10* signals is unclear, but it is known to interact with members of the TGF-β receptor family [31]. Further evidence of a potential role for *PEG10* in cell growth and carcinogenesis is that its expression can be regulated by the protooncogene *MYC* [29], by E2F transcription factors that modulate the cell cycle [32], and by the sex hormone androgen [33].

Although the exact mechanism of *PEG10* function in tumors remains to be elucidated, and the findings in this study must be verified in future studies using a larger sample number, the data presented in this work suggest a role for amplification and overexpression of *PEG10* in hepatocarcinogenesis.

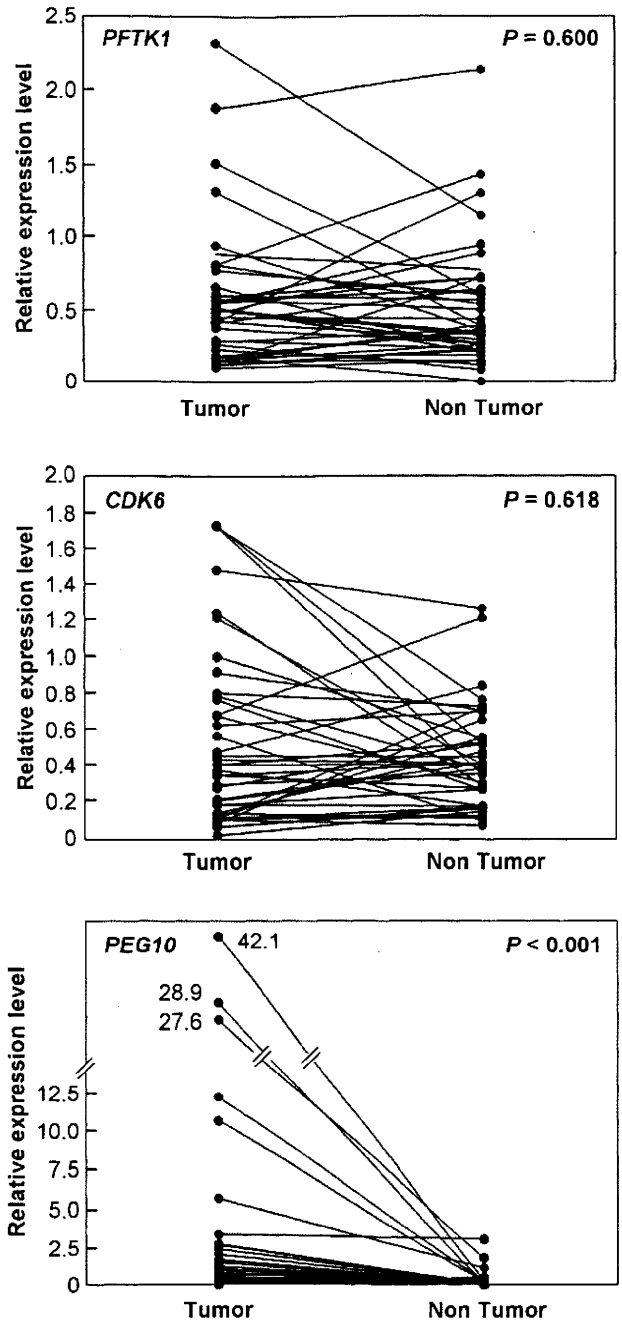


Fig. 4. Expression levels of *CDK14* (previously *PFTK1*), *CDK6*, and *PEG10* in paired tumor (T) and nontumor tissues (NT) from 41 patients with primary HCC. The expression level of each gene was determined as already described (for Fig. 2B). *CDK14*, *CDK6*, and *PEG10* were overexpressed in 22 (54%), 21 (51%), and 30 (73%) of the 41 tumors, respectively, compared with their nontumorous counterparts.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research (No. 20590408) from the Japan Society for the Program of Science (to K.Y.).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.cancergencyto.2010.01.004.

References

[1] Bosch FX, Ribes J, Cléries R, Díaz M. Epidemiology of hepatocellular carcinoma. Clin Liver Dis 2005;9:191–211.

[2] Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. Nat Genet 2002;31:339–46.

[3] Mei R, Galipeau PC, Prass C, Berno A, Ghandour G, Patil N, Wolff RK, Chee MS, Reid BJ, Lockhart DJ. Genome-wide detection of allelic imbalance using human SNPs and high-density DNA arrays. Genome Res 2000;10:1126–37.

[4] Zhao X, Li C, Paez JG, Chin K, Jänne PA, Chen TH, Girard L, Minna J, Christiani D, Leo C, Gray JW, Sellers WR, Meyerson M. An integrated view of copy number and allelic alterations in the cancer genome using single nucleotide polymorphism arrays. Cancer Res 2004;64:3060–71.

[5] Knuutila S, Björkqvist AM, Autio K, Tarkkanen M, Wolf M, Monni O, Szymanska J, Larramendy ML, Tapper J, Pere H, El-Rifai W, Hemmer S, Wasenius VM, Vidgren V, Zhu Y. DNA copy number amplifications in human neoplasms: review of comparative genomic hybridization studies. Am J Pathol 1998;152:1107–23.

[6] Sy SM, Wong N, Lai PB, To KF, Johnson PJ. Regional over-representations on chromosomes 1q, 3q and 7q in the progression of hepatitis B virus-related hepatocellular carcinoma. Mod Pathol 2005;18:686–92.

[7] Nakao K, Shibusawa M, Ishihara A, Yoshizawa H, Tsunoda A, Kusano M, Kurose A, Makita T, Sasaki K. Genetic changes in colorectal carcinoma tumors with liver metastases analyzed by comparative genomic hybridization and DNA ploidy. Cancer 2001;91:721–6.

[8] Strohmeyer DM, Berger AP, Moore DH 2nd, Bartsch G, Klocker H, Carroll PR, Loening SA, Jensen RH. Genetic aberrations in prostate carcinoma detected by comparative genomic hybridization and microsatellite analysis: association with progression and angiogenesis. Prostate 2004;59:43–58.

[9] García JL, Hernandez JM, Gutiérrez NC, Flores T, González D, Calasanz MJ, Martínez-Climent JA, Piris MA, Lopéz-Capitán C, González MB, Odero MD, San Miguel JF. Abnormalities on 1q and 7q are associated with poor outcome in sporadic Burkitt's lymphoma: a cytogenetic and comparative genomic hybridization study. Leukemia 2003;17:2016–24.

[10] Yen CC, Chen YJ, Chen JT, Hsia JY, Chen PM, Liu JH, Fan FS, Chiou TJ, Wang WS, Lin CH. Comparative genomic hybridization of esophageal squamous cell carcinoma: correlations between chromosomal aberrations and disease progression/prognosis. Cancer 2001;92:2769–77.

[11] Yang T, Chen JY. Identification and cellular localization of human PFTAIRE1. Gene 2001;267:165–72.

[12] Shu F, Lv S, Qin Y, Ma X, Wang X, Peng X, Luo Y, Xu BE, Sun X, Wu J. Functional characterization of human PFTK1 as a cyclin-dependent kinase. Proc Natl Acad Sci U S A 2007;104:9248–53.

[13] Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 1999;13:1501–12.

[14] Ono R, Kobayashi S, Wagatsuma H, Aisaka K, Kohda T, Kaneko-Ishino T, Ishino F. A retrotransposon-derived gene, *PEG10*, is a novel imprinted gene located on human chromosome 7q21. Genomics 2001;73:232–7.

[15] Okabe H, Satoh S, Furukawa Y, Kato T, Hasegawa S, Nakajima Y, Yamaoka Y, Nakamura Y. Involvement of PEG10 in human hepatocellular carcinogenesis through interaction with SIAH1. Cancer Res 2003;63:3043–8.

[16] Tsou AP, Chuang YC, Su JY, Yang CW, Liao YL, Liu WK, Chiu JH, Chou CK. Overexpression of a novel imprinted gene, *PEG10*, in human hepatocellular carcinoma and in regenerating mouse livers. J Biomed Sci 2003;10:625–35.

[17] Jia HL, Ye QH, Qin LX, Budhu A, Forgues M, Chen Y, Liu YK, Sun HC, Wang L, Lu HZ, Shen F, Tang ZY, Wang XW. Gene expression profiling reveals potential biomarkers of human hepatocellular carcinoma. Clin Cancer Res 2007;13:1133–9.

[18] Ip WK, Lai PB, Wong NL, Sy SM, Beheshti B, Squire JA, Wong N. Identification of *PEG10* as a progression related biomarker for hepatocellular carcinoma. Cancer Lett 2007;250:284–91.

[19] Luo JH, Ren B, Keryanov S, Tseng GC, Rao UN, Monga SP, Strom S, Demetris AJ, Nalesnik M, Yu YP, Ranganathan S, Michalopoulos GK. Transcriptomic and genomic analysis of human hepatocellular carcinomas and hepatoblastomas. Hepatology 2006; 44:1012–24.

[20] Inagaki Y, Yasui K, Endo M, Nakajima T, Zen K, Tsuji K, Minami M, Tanaka S, Taniwaki M, Itoh Y, Arii S, Okanoue T. *CREB3L4*, *INTS3*, and *SNAPAP* are targets for the 1q21 amplicon frequently detected in hepatocellular carcinoma. Cancer Genet Cytogenet 2008;180:30–6.

[21] Zen K, Yasui K, Nakajima T, Zen Y, Zen K, Gen Y, Mitsuyoshi H, Minami M, Mitsufuji S, Tanaka S, Itoh Y, Nakanuma Y, Taniwaki M, Arii S, Okanoue T, Yoshikawa T. *ERK5* is a target for gene amplification at 17p11 and promotes cell growth in hepatocellular carcinoma by regulating mitotic entry. Genes Chromosomes Cancer 2009;48:109–20.

[22] Kennedy GC, Matsuzaki H, Dong S, Liu WM, Huang J, Liu G, Su X, Cao M, Chen W, Zhang J, Liu W, Yang G, Di X, Ryder T, He Z, Surti U, Phillips MS, Boyce-Jacino MT, Fodor SP, Jones KW. Large-scale genotyping of complex DNA. Nat Biotechnol 2003;21:1233–7.

[23] Nannya Y, Sanada M, Nakazaki K, Hosoya N, Wang L, Hangaishi A, Kurokawa M, Chiba S, Bailey DK, Kennedy GC, Ogawa S. A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. Cancer Res 2005;65:6071–9.

[24] Gen Y, Yasui K, Zen K, Nakajima T, Tsuji K, Endo M, Mitsuyoshi H, Minami M, Itoh Y, Tanaka S, Taniwaki M, Arii S, Okanoue T, Yoshikawa T. A novel amplification target, *ARHGAP5*, promotes cell spreading and migration by negatively regulating RhoA in Huh-7 hepatocellular carcinoma cells. Cancer Lett 2009;275:27–34.

[25] Collins C, Rommens JM, Kowbel D, Godfrey T, Tanner M, Hwang SI, Polikoff D, Nonet G, Cochran J, Myambo K, Jay KE, Froula J, Cloutier T, Kuo WL, Yaswen P, Dairkee S, Giovanola J, Hutchinson GB, Isola J, Kallioniemi OP, Palazzolo M, Martin C, Ericsson C, Pinkel D, Albertson D, Li WB, Gray JW. Positional cloning of *ZNF217* and *NABC1*: genes amplified at 20q13.2 and overexpressed in breast carcinoma. Proc Natl Acad Sci U S A 1998;95: 8703–8.

[26] Pang EY, Bai AH, To KF, Sy SM, Wong NL, Lai PB, Squire JA, Wong N. Identification of PFTAIRE protein kinase 1, a novel cell division cycle-2 related gene, in the motile phenotype of hepatocellular carcinoma cells. Hepatology 2007;46:436–45.

[27] Hu C, Xiong J, Zhang L, Huang B, Zhang Q, Li Q, Yang M, Wu Y, Wu Q, Shen Q, Gao Q, Zhang K, Sun Z, Liu J, Jin Y, Tan J. PEG10 activation by co-stimulation of CXCR5 and CCR7 essentially contributes to resistance to apoptosis in $CD19^{+}CD34^{+}$ B cells from patients with B cell lineage acute and chronic lymphocytic leukemia. Cell Mol Immunol 2004;1:280–94.

[28] Kainz B, Shehata M, Bilban M, Kienle D, Heintel D, Krömer-Holzinger E, Le T, Kröber A, Heller G, Schwarzinger I, Demirtas D, Chott A, Döhner H, Zöchbauer-Müller S, Fonatsch C, Zielinski C, Stilgenbauer S, Gaiger A, Wagner O, Jäger U. Overexpression of the paternally expressed gene 10 (*PEG10*) from the imprinted locus on chromosome 7q21 in high-risk B-cell chronic lymphocytic leukemia. Int J Cancer 2007;121:1984–93.

[29] Li CM, Margolin AA, Salas M, Memeo L, Mansukhani M, Hibshoosh H, Szabolcs M, Klinakis A, Tycko B. *PEG10* is a c-MYC target gene in cancer cells. Cancer Res 2006;66:665–72.

[30] Ono R, Nakamura K, Inoue K, Naruse M, Usami T, Wakisaka-Saito N, Hino T, Suzuki-Migishima R, Ogonuki N, Miki H, Kohda T, Ogura A, Yokoyama M, Kaneko-Ishino T, Ishino F. Deletion of *Peg10*, an imprinted gene acquired from a retrotransposon, causes early embryonic lethality. Nat Genet 2006;38:101–6.

[31] Lux A, Beil C, Majety M, Barron S, Gallione CJ, Kuhn HM, Berg JN, Kioschis P, Marchuk DA, Hafner M. Human retroviral *gag*- and *gag-pol*-like proteins interact with the transforming growth factor-β receptor activin receptor-like kinase 1. J Biol Chem 2005;280: 8482–93.

[32] Wang C, Xiao Y, Hu Z, Chen Y, Liu N, Hu G. PEG10 directly regulated by E2Fs might have a role in the development of hepatocellular carcinoma. FEBS Lett 2008;582:2793–8.

[33] Jie X, Lang C, Jian Q, Chaoqun L, Dehua Y, Yi S, Yanping J, Luokun X, Qiuping Z, Hui W, Feili G, Boquan J, Youxin J, Jinquan T. Androgen activates PEG10 to promote carcinogenesis in hepatic cancer cells. Oncogene 2007;26:5741–51.

Hepatology Research 2010; 40: 654–660 doi: 10.1111/j.1872-034X.2010.00641.x

Case Report

Relapse of hepatitis C in a pegylated-interferon-α-2b plus ribavirin-treated sustained virological responder

Hideki Fujii,[1] Yoshito Itoh,[2] Naoki Ohnishi,[1] Masafumi Sakamoto,[1] Tohru Ohkawara,[1] Yoshihiko Sawa,[1] Koichi Nishida,[1] Takeshi Nishimura,[2] Kanji Yamaguchi,[2] Kohichiroh Yasui,[2] Masahito Minami,[2] Takeshi Okanoue,[3] Yasuo Ohkawara[1] and Toshikazu Yoshikawa[2]

[1]*Department of Internal Medicine, Aiseikai Yamashina Hospital,* [2]*Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Graduate School of Medical Science, Kyoto, and* [3]*Division of Gastroenterology, Saiseikai Suita Hospital, Osaka, Japan*

A 41-year-old woman with chronic hepatitis C was treated with pegylated-interferon (PEG-IFN)-α-2b plus ribavirin for 24 weeks. She had hepatitis C virus (HCV) genotype 2a (1600 KIU/mL), and her liver histology showed mild inflammation and fibrosis. Four weeks after the start of the therapy, she achieved a rapid virological response (RVR) and then a sustained virological response (SVR). Serum alanine aminotransferase (ALT) levels remained within normal ranges and HCV RNA continued to be negative. However, ALT levels flared with the re-emergence of HCV RNA in the serum 1.5 years after discontinuation of therapy. HCV RNA obtained from sera before therapy and after relapse shared a 98.6% homology with the E2 region, and phylogenetic analyses indicated that they were the same HCV strain. These results eliminated the possibility of a re-infection and strongly indicated a late relapse of the disease. Therefore, follow-up is necessary for chronic hepatitis C patients after SVR, even if they respond well to therapy, including RVR.

Key words: chronic hepatitis C, genotype 2a, sustained virological response, relapse, phylogenetic analyses.

INTRODUCTION

HEPATITIS C VIRUS (HCV) is an important cause of chronic liver disease, and more than 170 million people are infected worldwide, including 1.5–2 million people in Japan.[1] Approximately 70% of Japanese chronic hepatitis C patients are infected with genotype 1b, whereas the rest are infected with genotypes 2a or 2b.[2] At present, pegylated-interferon (PEG-IFN)-α plus ribavirin is the optimal therapy for chronic hepatitis C. Sustained virological response (SVR), defined as undetectable serum HCV RNA 24 weeks after therapy completion, is the primary goal of this therapy. Approximately 80% of patients infected with genotypes 2 or 3 achieve SVR after 24 weeks of treatment, whereas approximately 50% patients with genotype 1 achieve SVR after 48 weeks of treatment.

Late relapse, defined as a HCV RNA reappearance in serum after achieving SVR, is rare in SVR patients. Furthermore, distinguishing relapse from re-infection is difficult without comparing the HCV nucleotide sequence before the start of the therapy and after relapse. Here we describe the clinical course of an HCV genotype 2a-infected woman treated with PEG-IFN-α plus ribavirin for 24 weeks. She achieved a rapid virological response (RVR) because HCV RNA was undetectable by a qualitative polymerase chain reaction (PCR) assay 4 weeks after initiating therapy. However, she achieved SVR and suffered a relapse of chronic hepatitis C 1.5 years after therapy discontinuation. We analyzed nucleotide sequences within the E2 region of HCV RNA containing the hypervariable region (HVR)1 and the IFN sensitivity-determining region (ISDR) of nonstructural protein 5A (NS5A), using sera before treatment and after relapse and confirmed that they were the same HCV strain.

Correspondence: Dr Yoshito Itoh, Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kawaramachi-Hirokoji, Kamigyou-ku, Kyoto 602-8566, Japan. Email: yitoh@koto.kpu-m.ac.jp
Received 15 September 2009; revision 23 November 2009; accepted 6 December 2009.

 © 2010 The Japan Society of Hepatology

CASE REPORT

A 41-YEAR-OLD WOMAN had elevated serum alanine aminotransferase (ALT; 138 IU/L) and aspartate aminotransferase (AST; 248 IU/L) levels on a routine medical check-up in mid-September 2005. Because of liver dysfunction in October 2005, she visited Aiseikai Yamashina General Hospital for further examination. She had no family history of liver diseases. Her height was 145 cm and weight was 42 kg. No abnormalities were detected on physical examination; her average alcohol intake was less than 20 g/week. She had no history of i.v. drug abuse.

Table 1 shows the laboratory data. On 25 October 2005, transaminase and biliary enzyme levels were elevated. Serum anti-HCV antibody was positive. The HCV RNA load was 2400 KIU/mL (Amplicor Monitor ver. 2.0; Roche Diagnostic Systems, Tokyo, Japan), and she had the 2a HCV genotype. She hesitated to undergo IFN treatment in the beginning. We strictly prohibited her from alcohol and started treating her with 600 mg/day oral ursodeoxycholic acid (UDCA) and 40 mL i.v. glycyrrhizin twice a week (Stronger Neo-Minophagen C, SNMC). Her liver functions improved significantly, but did not normalize following treatment with UDCA and SNMC.

She was admitted to Aiseikai Yamashina General Hospital on 3 February 2006, and PEG-IFN plus ribavirin treatment was initiated for chronic hepatitis C. Abdominal ultrasonography revealed that the liver was almost normal in size, the edge was sharp and the internal echo was slightly coarse. Other tests including hepatitis B virus PCR, HBc antibody, HIV 1/2 antibodies, antinuclear antibody, anti-mitochondrial antibody, serum ceruloplasmin, copper and ferritin were normal. The laboratory test results obtained on 3 February 2006 are presented in Table 1. The liver biopsy specimen before treatment revealed mild fibrosis with mild inflammation, which was graded as A1F1 according to the classification of Ichida *et al.* or Bedossa and Poynard.[3,4] She received combination therapy consisting of PEG-IFN-α-2b (1.5 μg/kg; 60 μg) once a week plus 600 mg ribavirin daily.

After therapy initiation, ALT levels declined rapidly and remained within the normal range after completion of the treatment. Serum HCV RNA levels were measured by a quantitative PCR assay (Amplicor HCV Monitor ver. 2.0) before therapy initiation and after relapse and by a qualitative PCR assay (Amplicor HCV Test ver. 2.0) at 4, 8, 12, 16, 20 and 24 weeks (all during the treatment period) as well as at 4, 8, 12, 16, 20 and 24 weeks after therapy completion. Serum HCV RNA was qualitatively

Table 1 Laboratory findings

	Normal	Initial visit (10/20/2005)	Before PEG-IFN + Rib (2/3/2006)	After relapse (1/25/2008)
White blood cell (μL)	(3900–9300)	9070	8110	8800
Red blood cell ($\times 10^4$/μl)	(425–571)	458	433	412
Platelet ($\times 10^4$/μL)	(12.7–35.6)	29.2	29.7	27.3
PT (%)		85%	82%	92%
Albumin (g/dL)	(4.0–5.0)	4.2	4.2	4.1
T. Bil (mg/dL)	(0.3–1.2)	0.6	0.4	0.4
AST (IU/I)	(<33)	87	35	61
ALT (IU/I)	(<35)	195	38	96
ALP (IU/I)	(115–360)	278	240	247
γ-GTP (IU/I)	(<47)	256	48	88
RPR	(–)	NA	(–)	(–)
HBsAg	(–)	(–)	(–)	(–)
ANA	(<40)	NA	<40	<40
Type IV collagen 7S (ng/mL)	(<5)	NA	3.8	3.2
Serum ferritin (ng/mL)	(5.3–179.7)	NA	50.7	NA
HCV RNA (Amplicor Monitor ver. 2.0) (KIU/mL)	(–)	2400	1600	2600
HCV genotype		2a	2a	2a

ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANA, antinuclear antibody; AST, aspirate aminotransferase; γ-GTP, γ-glutamyltranspeptidase; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; NA, not available; PT, prothrombin time; RPR, Rapid Plasma Reagin; T. Bil., total bilirubin.

© 2010 The Japan Society of Hepatology

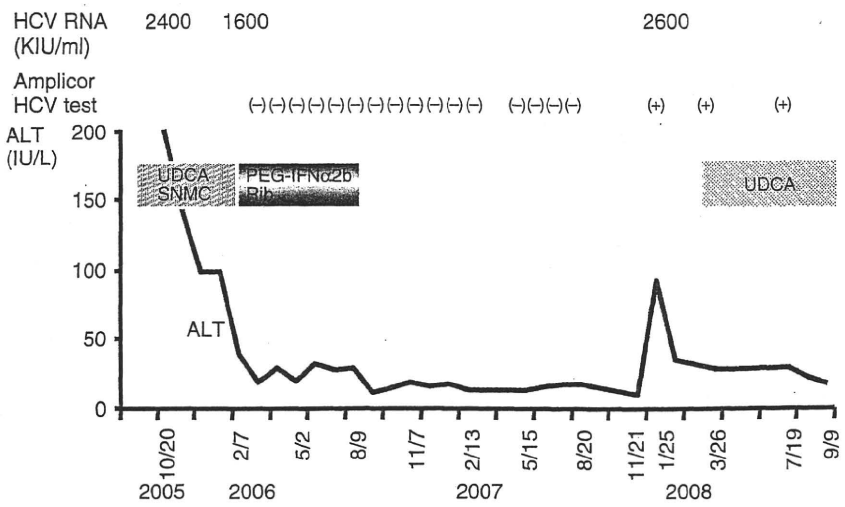


Figure 1 Levels of alanine aminotransferase (ALT) and hepatitis C virus (HCV) RNA load during the clinical course. Pegylated-interferon (PEG-IFN)-α plus ribavirin combination therapy was started in February 2006 and continued for 24 weeks until August 2006. HCV RNA was undetectable within 4 weeks. ALT levels remained within normal ranges until November 2007. Relapse occurred in January 2008. SNMC, Stronger Neo-Minophagen C; UDCA, ursodeoxycholic acid.

undetectable 4 weeks after therapy initiation and remained undetectable 6 months after therapy completion. The patient had few side-effects, and the treatment was completed without reducing either of the drugs.

After achieving SVR, she underwent monthly liver function tests, and a qualitative PCR assay was performed occasionally. In December 2007, her liver function tests deteriorated. AST/ALT levels were elevated, and she tested positive for HCV RNA on 25 January 2008 (Fig. 1, Table 1). A quantitative PCR assay indicated that the HCV RNA titer was 2600 IU/L, and the HCV genotype was 2a. The patient again started taking 600 mg UDCA daily, and ALT returned to low levels (~30–40 IU/L). Repeated tests showed that HCV RNA was persistently positive.

To determine if HCV RNA that appeared 1.5 years after treatment completion was identical to that before therapy, we compared the nucleotide sequences of the two coding regions, namely, the E2 region containing HVR1 and ISDR of NS5A. Informed consent was obtained from the patient before analysis, and the serum samples obtained before treatment and after relapse were stored at −80 °C until use.

Virological analyses proceeded as follows. To reconfirm HCV genotyping, direct sequencing of the 5′-untranslated region was performed, as described previously.[5,6] The genotypes were classified according to the nomenclature proposed in a previous report and were determined to be 2a in both the samples. HCV RNA was amplified by reverse transcription (RT)–PCR to directly sequence the E2 and ISDR regions.

In brief, RNA was extracted from 140 μL sera using a commercially available kit (QIAamp viral RNA kit; QIAGEN, Valencia, CA, USA) and dissolved in 50 μL diethylpyrocarbonate-treated water. This sample was used for RT with random hexamer primers (SuperScript III First-Strand Synthesis System for RT–PCR cDNA synthesis kit; Invitrogen, Carlsbad, CA, USA). The E2 region was amplified by nested PCR, and ISDR regions were amplified by hemi-nested PCR. Each 50-μL PCR reaction contained 100 nM of each primer, 1 ng template cDNA, 5 μL 10× Ex Taq buffer, 4 μL deoxyribonucleotide triphosphate mixture, and 1.25 U of Takara Ex Taq HS (Takara Ex Taq, Otsu, Japan).

The PCR primers were set based on a reference HCV sequence (accession no. AF177036).

The first PCR primer sequences for E2 were: sense (1422, 1441) 5′-ACTTCTCTATGCAGGGAGCG-3′ and antisense (2437, 2418) 5′-GTTTTGGTGGAGGTGGAG AA-3′; and sense (2171, 2190) 5′-TGCCTGATCGACTA CCCCTA-3′ and antisense (2730, 2711) 5′-AGGCC AGTGAGGGAATAGGT-3′. The second PCR primer sequences for E2 were: sense (1453, 1472) 5′-CGTT GTCATCCTTCTGTTGG-3′ and antisense (2261, 2242) 5′-CAACCCCTCCCACATACATC-3′; and sense (2189, 2208) 5′-TACAGGCTCTGGCATTACCC-3′ antisense (2698, 2679) 5′-TACCCGACCCTTGATGTACC-3′.

© 2010 The Japan Society of Hepatology

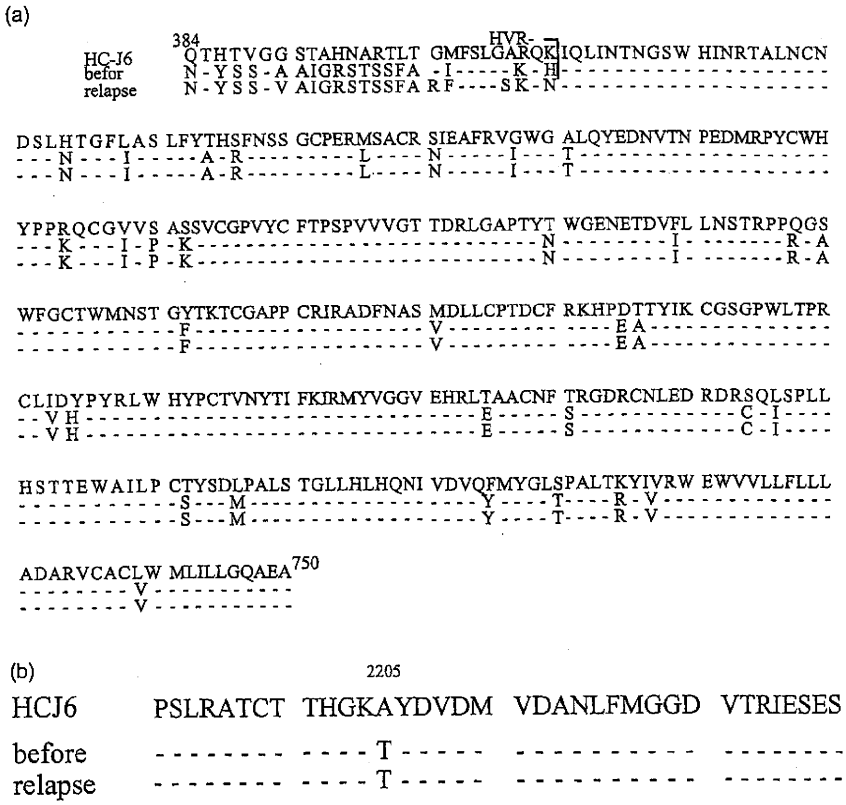


Figure 2 (a) Comparison of the hepatitis C virus (HCV) E2 (top) region amino acid sequences. Sequencing was performed for serum samples obtained before therapy on 3 February 2006 and after relapse on 25 January 2008. Sequences were aligned against the HCJ6 HCV genotype 2a reference sequence (GenBank accession no. D00944). Hypervariable region (HVR)1 positions of E2 are indicated by numbers corresponding to the amino acid positions within the HCV genotype 2a polyprotein of the reference sequence. There were five amino acid mutations in these regions between the two samples. (b) IFN sensitivity-determining region (ISDR) sequences before therapy and after relapse showed the same single mutation at codon 2205. Sequences were aligned against the HCJ6 HCV genotype 2a reference sequence (GenBank accession no. D00944). ISDR positions are indicated as numbers corresponding to the amino acid positions within the HCV genotype 2a polyprotein of the reference sequence.

The first PCR primer sequences for ISDR were: sense (6866, 6885) 5′-ACGTCCATGCTAACAGACCC-3′ and antisense (7185, 7166) 5′-GGGAATCTCTTCTTGGG GAG-3′. The second PCR primer sequences for ISDR were the sense primer from the first-round PCR and a new antisense primer (7109, 7090) 5′-CGAGAG AGTCCAGAACGACC-3′.[7]

Polymerase chain reaction products were separated by electrophoresis on 1% or 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light. The products were purified and sequenced with second-round PCR primers, using a dye terminator sequencing kit (BigDye Terminator ver. 1.1 cycle sequencing kit; Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 310 genetic analyzer (Applied Biosystems). Sequence alignments and phylogenetic analyses were performed with MEGA4 software.[8] Nucleotide sequences obtained from the two samples were compared to 23 published HCV genotype 2a sequences. A phylogenetic tree was constructed by the neighbor-joining method[9] based on the nucleotide sequence of the E2 region, with pairwise distances being estimated using the Kimura two-parameter method. Bootstrap values were determined on 1000 re-samplings of data sets.[10]

The E2 nucleotide sequences before treatment and after relapse were 98.6% similar. Except HVR1, two of the samples were 99.0% similar. The amino acid sequences in the E2 region, except HVR1, were identical between the two samples. There was a difference of five amino acids in HVR1 (Fig. 2a).

When compared to known HCV isolates of various genotypes whose entire coding region sequence has

© 2010 The Japan Society of Hepatology

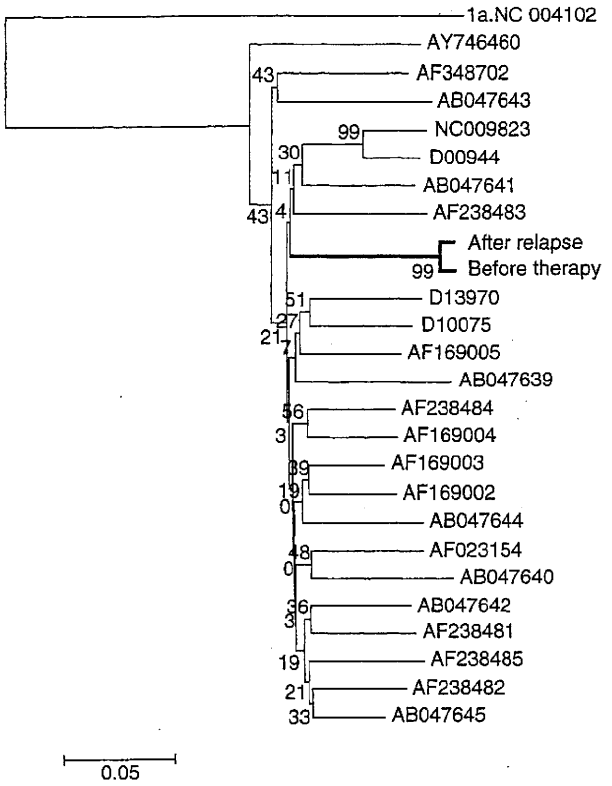


Figure 3 A phylogenetic tree was constructed by the neighbor-joining method based on the nucleotide sequence of the E2 region (1101 nt) of 23 genotype 2a strains using the genotype 1a hepatitis C virus (HCV) isolate (NC004102) as an outgroup. The isolates obtained in the present study before therapy and after relapse are indicated in bold letters for clarity. Twenty-three reported genotype 2a HCV isolates, whose entire coding region sequence is known, are included for comparison, and their accession numbers are shown. Bootstrap values were determined on 1000 re-samplings of datasets.

been determined, the two isolates were the closest to a particular genotype 2a HCV isolate (accession no. AB047645) with 87.3% nucleotide sequence similarity; however, the isolates were only 64.4–72.7% similar to known genotype non 2a (1a NC004102, 1b D90208, 2b D01221, 3a D17763, 4a Y11604, 5a Y13184 and 6a Y12083). The phylogenetic tree of 23 genotype 2a HCV isolates, constructed based on the E2 1101-nucleotide sequence, indicated that the sample obtained after relapse bifurcated from a common trunk with the sample before treatment, and we confirmed that these two samples were the closest to each other among all known genotype 2a HCV isolates (Fig. 3). The results of sequencing analysis before therapy and after re-emergence of viremia ruled out the possibility of a re-infection and strongly suggested a late relapse of chronic hepatitis C.

Interferon sensitivity-determining region sequences before treatment and after relapse showed 98.9% similarity. The amino acid sequences of the two ISDR regions were completely identical. The sequences of the HCJ6 (accession no. D00944) strain were defined as wild-type ISDR, and those that deviated from this strain were defined as the mutant type. ISDR sequences before treatment and after relapse were different in only one codon (2205) when compared with the reference HCJ6 sequence (Fig. 2b).

DISCUSSION

IN THIS STUDY, we clarified that E2 1101-nucleotide sequences of HCV isolated from sera before treatment and after relapse shared a 98.6% homology. Furthermore, phylogenetic analyses classified these two samples as the same strain. These results ruled out the possibility of a re-infection and strongly suggested a late relapse of chronic hepatitis C.

Hepatitis C virus is an RNA virus belonging to the genus *Hepacivirus* in the Flaviviridae family. Similar to other RNA viruses, HCV circulates as a genetically distinct population, demonstrating a quasispecies.[11] HCV HVR1, which is composed of 27 amino acids and is located at the 5′ terminus of the E2 gene, is highly variable among and within infected patients,[12–14] so it can be used to identify individual HCV isolates.[15,16] HCV HVR1 changes rapidly over time in the same individual. Our pairwise sequences were not completely identical but shared a high homology, which was equal to the homology reported previously.[16] These results suggest that the patient achieved SVR but suffered a relapse of hepatitis C after 1.5 years.

Some reports have indicated that in a majority of patients with SVR, low-level HCV RNA can be detected in lymphocytes, monocytes/macrophages and liver, despite constantly undetectable HCV RNA in sera.[17–19] This "occult" persistence of HCV replication could potentially play a role in late recurrence after treatment. However, the significance/mechanism of HCV RNA persistence in the liver or peripheral blood mononuclear cells is still uncertain, and data regarding occult persistence are conflicting.[20] Moreover, it is unclear as to how many of these late relapse patients were "true" relapsers and how many were re-infected. The relapse rates after SVR in IFN monotherapy are approximately 5–10%.[21,22] Nakayama *et al.* recently reported a late relapse of

© 2010 The Japan Society of Hepatology

hepatitis C after IFN-α plus ribavirin therapy and summarized late relapsing cases in Japan.[23] They indicated that compared to reports from foreign countries, late relapses were very rare in Japan, particularly after IFN and ribavirin therapy and that the relapse interval was principally restricted to within 2 years after therapy completion. Four hundred and fifty-five chronic hepatitis C patients were cured by PEG-IFN plus ribavirin therapy in the study group of Kyoto Prefectural University of Medicine and related hospitals, and this is the only case of late relapse to date (Itoh Y. *et al.*, 2009 unpublished data). This may be the first reported case of relapse after SVR with PEG-IFN plus ribavirin therapy.

Several host and viral characteristics are associated with the likelihood of response to IFN-based therapy. The HCV genotype and viral load are the most important viral predictors, and the ISDR sequence variation[24] and substitutions of amino acids 70 and/or 91 in the core region[25] within the HCV genome have been recently advocated in patients with genotype 1. It is interesting to note that only one amino acid varied in ISDR compared to the reference sequence in our case. For patients with HCV genotype 2a, Hayashi *et al.* reported that ISDR amino acid variations compared to the reference sequence and RVR as well as negative HCV at 4 weeks are important predictors of SVR in PEG-IFN monotherapy.[7] ISDR interacts with interferon-inducible double-stranded RNA-activated protein kinase (PKR) and inactivates HCV replication *in vitro*.[26] According to the report by Hayashi *et al.*,[7] an A-to-T mutation at codon 2205 (Fig. 2b) can be interpreted as wild type, and hence ISDR in this case contained no mutations, which may have influenced HCV RNA re-emergence after achieving SVR.

Patients with RVR, defined as a negative HCV RNA at 4 weeks, are more likely to have SVR.[7,27] In our case, HCV RNA was negative at 4 weeks, which indicated that this case may be cured; however, relapse of hepatitis C occurred after 1.5 years. The data concerning the efficacy of re-treatment of genotype 2 chronic hepatitis C are limited. According to the report by Moucari *et al.*,[28] there is a higher rate of SVR in genotype non-1 relapsers. Therefore, our patient could be retreated with a second PEG-IFN plus ribavirin combination therapy. However, because the patient is 41 years old and has stage F1 hepatic fibrosis, we will recommend that she wait for a new drug such as a protease inhibitor. Further research for unknown factors to predict late relapse after achieving SVR might be necessary.

In conclusion, SVR patients may have a potential risk of HCV reactivation. Annual surveillance including HCV RNA testing seems clinically reasonable for detecting spontaneous relapse and recurrence of hepatitis C in SVR patients.

REFERENCES

1 National Institutes of Health Consensus Development Conference Statement: Management of hepatitis C: 2002 – June 10–12, 2002. *Hepatology* 2002; 36: S3–20.

2 Hayashi J, Kishihara Y, Yamaji K *et al.* Transmission of hepatitis C virus by health care workers in a rural area of Japan. *Am J Gastroenterol* 1995; 90: 794–9.

3 Ichida F, Tsuji T, Omata M *et al.* New Inuyama classification: new criteria for histological assessment of chronic hepatitis. *Internat Hepatol Comm* 1996; 6: 112–9.

4 Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology* 1996; 24: 289–93.

5 Germer JJ, Rys PN, Thorvilson JN, Persing DH. Determination of hepatitis C virus genotype by direct sequence analysis of products generated with the Amplicor HCV test. *J Clin Microbiol* 1999; 37: 2625–30.

6 Gargiulo F, De Francesco MA, Pinsi G *et al.* Determination of HCV genotype by direct sequence analysis of quantitative PCR products. *J Med Virol* 2003; 69: 202–6.

7 Hayashi K, Katano Y, Honda T *et al.* Mutations in the interferon sensitivity-determining region of hepatitis C virus genotype 2a correlate with response to pegylated-interferon-alpha 2a monotherapy. *J Med Virol* 2009; 81: 459–66.

8 Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 2007; 24: 1596–9.

9 Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987; 4: 406–25.

10 Felsenstein J. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 1985; 39: 783–91.

11 Martell M, Esteban JI, Quer J *et al.* Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: quasispecies nature of HCV genome distribution. *J Virol* 1992; 66: 3225–9.

12 Ogata N, Alter HJ, Miller RH, Purcell RH. Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. *Proc Natl Acad Sci USA* 1991; 88: 3392–6.

13 Weiner AJ, Geysen HM, Christopherson C *et al.* Evidence for immune selection of hepatitis C virus (HCV) putative envelope glycoprotein variants: potential role in chronic HCV infections. *Proc Natl Acad Sci USA* 1992; 89: 3468–72.

14 Okamoto H, Kojima M, Okada S *et al.* Genetic drift of hepatitis C virus during an 8.2-year infection in a chimpanzee: variability and stability. *Virology* 1992; 190: 894–9.

15 Lee WM, Polson JE, Carney DS, Sahin B, Gale M Jr. Reemergence of hepatitis C virus after 8.5 years in a patient with

© 2010 The Japan Society of Hepatology

hypogammaglobulinemia: evidence for an occult viral reservoir. *J Infect Dis* 2005; 192: 1088–92.
16 Nakayama H, Sugai Y, Ikeya S, Inoue J, Nishizawa T, Okamoto H. Molecular investigation of interspousal transmission of hepatitis C virus in two Japanese patients who acquired acute hepatitis C after 40 or 42 years of marriage. *J Med Virol* 2005; 75: 258–66.
17 Castillo I, Rodriguez-Inigo E, Lopez-Alcorocho JM, Pardo M, Bartolome J, Carreno V. Hepatitis C virus replicates in the liver of patients who have a sustained response to antiviral treatment. *Clin Infect Dis* 2006; 43: 1277–83.
18 Pham TN, MacParland SA, Mulrooney PM, Cooksley H, Naoumov NV, Michalak TI. Hepatitis C virus persistence after spontaneous or treatment-induced resolution of hepatitis C. *J Virol* 2004; 78: 5867–74.
19 Radkowski M, Gallegos-Orozco JF, Jablonska J *et al.* Persistence of hepatitis C virus in patients successfully treated for chronic hepatitis C. *Hepatology* 2005; 41: 106–14.
20 Welker MW, Zeuzem S. Occult hepatitis C: how convincing are the current data? *Hepatology* 2009; 49: 665–75.
21 McHutchison JG, Poynard T, Esteban-Mur R *et al.* Hepatic HCV RNA before and after treatment with interferon alone or combined with ribavirin. *Hepatology* 2002; 35: 688–93.
22 Veldt BJ, Saracco G, Boyer N *et al.* Long term clinical outcome of chronic hepatitis C patients with sustained virological response to interferon monotherapy. *Gut* 2004; 53: 1504–8.
23 Nakayama H, Ojima T, Kusano M, Endo K, Takahashi M, Sugai Y. Two cases of chronic hepatitis C with sustained virological response in whom serum HCV RNA reappeared two or twelve years after the end of IFN treatment. *Kanzo* 2006; 47: 550–7. (In Japanese).
24 Enomoto N, Sakuma I, Asahina Y *et al.* Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996; 334: 77–81.
25 Akuta N, Suzuki F, Sezaki H *et al.* Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. *Intervirology* 2005; 48: 372–80.
26 Gale M Jr, Blakely CM, Kwieciszewski B *et al.* Control of PKR protein kinase by hepatitis C virus nonstructural 5A protein: molecular mechanisms of kinase regulation. *Mol Cell Biol* 1998; 18: 5208–18.
27 Shiffman ML, Suter F, Bacon BR *et al.* Peginterferon alfa-2a and ribavirin for 16 or 24 weeks in HCV genotype 2 or 3. *N Engl J Med* 2007; 357: 124–34.
28 Moucari R, Ripault MP, Oules V *et al.* High predictive value of early viral kinetics in retreatment with peginterferon and ribavirin of chronic hepatitis C patients non-responders to standard combination therapy. *J Hepatol* 2007; 46: 596–604.

© 2010 The Japan Society of Hepatology

日消誌　2011；108：202―209

今月のテーマ●B型慢性肝炎に対する最新の治療

ラミブジンとアデホビル併用不応例に対するアデホビルとエンテカビル併用療法

小関　至　木村睦海　荒川智宏
中島知明　桑田靖昭　赤池　淳
大村卓味　佐藤隆啓　狩野吉康
豊田成司[1)]

要旨：ラミブジン（LAM）とアデホビル（ADV）併用療法を12カ月以上行い，HBV DNAが3log copies/ml以上を示したB型慢性肝疾患18例を対象とし，48週以上ADVとエンテカビル（ETV）の併用療法を行いウイルス動態についての検討を行った．LAM耐性例，ADV耐性例，ETV耐性例，多剤耐性例はそれぞれ100%，27.8%，33.3%，55.6%であった．平均HBV DNAはベースラインで4.1log copies/mlより48週の時点で2.9log copies/mlと低下した．ETV耐性を有する症例でHBV DNA減衰量は低下した．本併用療法による副作用は出現せず，48週の経過で新たに獲得したアナログ耐性は認めなかった．テノホビル（TDF）が使用できない本邦の現状ではLAMとADV併用不応例に対して，ADVとETV併用療法は試みるべき治療と思われた．

索引用語：ラミブジン，アデホビル，エンテカビル，B型肝炎ウイルス，耐性変異

はじめに

B型肝炎ウイルスによる持続感染の患者は世界で約3億5千万人いるといわれており[1)]，このウイルスによる持続感染はしばしば肝硬変，肝不全を惹起し，肝細胞癌の発生の原因となる[2)]．インターフェロン（interferon；IFN）製剤はB型肝炎ウイルスの増殖を抑制し，肝炎の鎮静に有効であるが，その効果は限定的であり，ペグインターフェロン（pegylated IFN；PEG-IFN）は30～40%の患者でsustained responseを達成するとされている[3)4)]が，本邦では現在治験中である．核酸アナログ製剤はB型肝炎ウイルスのDNAポリメラーゼを抑制してDNA合成を阻害し，ウイルス増殖を抑える薬剤であり，血液生化学検査値，肝組織所見の改善を促す[5)6)]．長期の核酸アナログ投与は肝硬変の進展や肝細胞癌の発生を抑制し，長期予後を改善する可能性が指摘されている[7)]．一方で長期に及ぶ核酸アナログ投与は薬剤に対する変異株の発生を促し，しばしば，ウイルス学的ブレイクスルーを引きおこす[8)]．実際に長期のラミブジン（lamivudine；LAM）投与は高率にLAM耐性ウイルスの出現を促した[8)9)]．近年登場した新規の核酸アナログ製剤であるエンテカビル（entecavir；ETV）はLAMと比較して耐性ウイルス

1）札幌厚生病院第3消化器科

Efficacy of entecavir and adefovir combination therapy in patients with chronic hepatitis B refractory to lamivudine and adefovir combination therapy

Itaru OZEKI, Mutsuumi KIMURA, Tomohiro ARAKAWA, Tomoaki NAKAJIMA, Yasuaki KUWATA, Jun AKAIKE, Takumi OHMURA, Takahiro SATO, Yoshiyasu KARINO and Joji TOYOTA[1)]
1）Department of Hepatology, Sapporo Kosei General Hospital
Corresponding author：小関　至（i.ozeki@ja-hokkaidoukouseiren.or.jp）

Table 1. 背景因子

性別		男性：13　女性：5
年齢（歳）	Mean+/−SD	59.6+/−9.0
肝硬変（あり）	(No. [%])	10 (55.6)
肝癌既往（あり）	(No. [%])	5 (27.8)
LAM+ADV 治療期間（月）	Mean+/−SD	29.1+/−13.1
HBV genotype		Bj：1　C：17
HBV DNA（$\log_{10}$ copies/m*l*）	Mean+/−SD	4.10+/−1.18
HBeAg（+）	(No. [%])	13 (72.2)
ALT（IU/L）	Mean+/−SD	35.9+/−17.7
LAM 耐性	(No. [%])	18 (100)
ADV 耐性	(No. [%])	5 (27.8)
ETV 耐性	(No. [%])	6 (33.3)
多剤耐性	(No. [%])	10 (55.6)

の出現が少なく，抗ウイルス作用が強いことがいくつかの臨床試験で明らかとされた[10)~12)]．本邦においてもETVは2007年の承認後LAMに代わり第一選択の核酸アナログ製剤となった．しかし，既に世界中で多くのLAM耐性患者を認めており，これらの症例に対して2009年の米国肝臓病学会（American Association for the Study of Liver Disease；AASLD）はアデホビル（adefovir dipivoxil；ADV），あるいはテノホビル（tenofovir disoproxil fumarate；TDF）のLAMとの併用投与，あるいはemtricitabine（FTC）とTDFの併用投与への切り替えを推奨した[13)]．同様にヨーロッパ肝臓病学会（European Association for the Study of the Liver；EASL）からはTDFの併用[14)]が，本邦からはADVの併用が推奨された[15)]．LAM耐性例に対するLAMとADV併用療法（以下LAM/ADV療法）による抗ウイルス効果の発現は緩徐であり，大多数のLAM耐性患者に有効であるが，少数例でHBV DNAの低下量が不十分であることが報告されている[16)17)]．今回われわれはLAM/ADV療法不応例に対するADVとETV療法（ADV/ETV療法）48週の成績を検討したので報告する．

I 対象と方法

LAM/ADV療法を少なくとも1年以上行い，HBV DNAが3log copies/m*l*（以下log）以上を示した18例を対象とした．自己免疫性肝炎，アルコール性肝障害，うっ血性肝障害の合併例，C型肝炎ウイルスあるいはヒト免疫不全ウイルスの併発例，黄疸・腹水・脳症・消化管出血をともなう患者は除外した．18例中6例はLAM耐性に対するETV投与の既往を有した．2名の患者がADV投与中に血清クレアチニン上昇をきたしたため，ADVは隔日投与が行われていた．

HBV DNAはTaqMan PCR法（Roche Diagnostics, Tokyo, Japan），耐性ウイルスの検討はINNO-LiPA HBV DR version 2, version 3（Innogenetics Gent, Belgium）を用いた[18)]．

2群の検定にはStudent's t test, Mann-Whitney U test, chi-squared test, Fisher's exact testを用い，$p<0.05$を有意とした．

II 結　果

18例の背景因子をTable 1に示す．5例で肝癌の既往を認め，1例はADV/ETV療法中に肝癌を発症したが，肝部分切除あるいは経皮的ラジオ波焼灼療法で根治的な治療を受けた．10例は代償性肝硬変の状態で，遺伝子型では1例がBj型，17例がC型を示し，HBe抗原陽性は13例（72.2%）であった．LAM耐性は18例全例（100%），ADV耐性は5例（27.8%），ETV耐性

Table 2. ベースライン，48週の時点におけるHBV DNA，HBe抗原，ALT値の推移とINNO-LiPA法によるベースラインのアナログ耐性

Case	HBV DNA (log copies/ml)			HBeAg (S/CO)		ALT (IU/L)		Resistance Mutation		
	0W	48W	0W-48W	0W	48W	0W	48W	LAM	ADV	ETV
1	7.6	3.1	4.5	1.7	3.4	74	39	+	+	
2	5.03	3.69	1.34	44	18	32	28	+		
3	3.09	1.8	1.29	—	—	31	16	+		+
4	4.12	2.51	1.61	245	106	27	20	+		+
5	4.9	4.6	0.3	528	359	49	36	+		+
6	3	1.8	1.2	3.7	—	27	28	+	+	
7	5.2	3.53	1.67	—	—	39	47	+		
8	3.87	2.93	0.94	1043	927	15	15	+		
9	4.93	3.91	1.02	87	39	28	25	+		+
10	5.24	4.17	1.07	161	121	48	43	+		
11	4.76	2.64	2.12	1.9	1.3	40	36	+		
12	3.46	3.36	0.1	—	—	37	30	+		
13	3	1.8	1.2	7.5	4.8	10	11	+	+	
14	3.61	2.51	1.1	3.9	3	28	25	+		
15	3.07	1.8	1.27	5.7	5.1	21	22	+		
16	3.96	3.11	0.85	164	94	73	138	+		+
17	3.17	1.8	1.37	—	—	28	42	+	+	
18	3.59	2.89	0.7	—	—	14	16	+	+	+

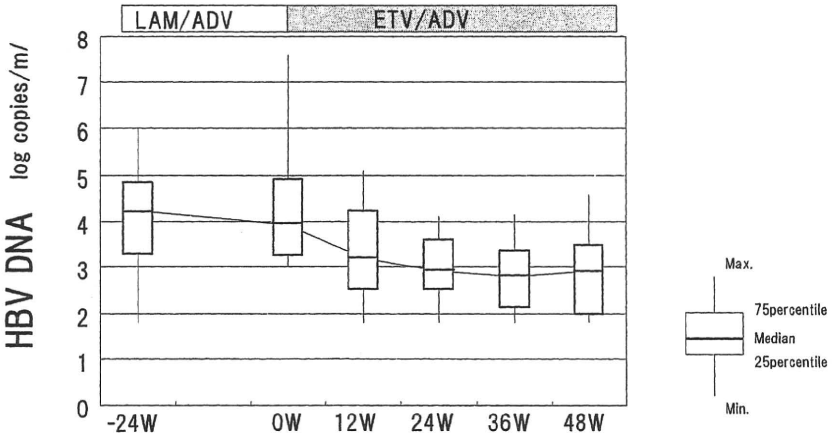


Figure 1. LAM/ADV療法・ADV/ETV療法によるHBV DNAの推移：HBV DNAはベースライン4.1log copies/mlから48週2.9log copies/mlと，48週で1.2log copies/ml低下した．

は6例（33.3%），10例（55.6%）は3剤耐性を認めた（Table 2）．

平均HBV DNAはADV/ETV療法にてベースライン4.1log，12週3.3log，24週3.0log，36週2.8log，48週2.9logと緩徐に低下した（Figure 1）．18例中5例が48週の治療中に2.1log未満を呈した．18例中13例は48週で1log以上のHBV DNA量の低下を示したが，残る5例の低下量は1log未満であった．HBe抗原陽性例・陰性例で治療48週のHBV DNA低下量に差を認めなかった．アナログ耐性別の治療48週でのHBV DNAの減衰量はLAM耐性で1.2log，LAM耐性＋