Cell Culture and Transfection. Huh-7 human HCC cells, U-2 OS human osteosarcoma cells, 293T human embryonic kidney cells, mouse lymph node cells, and P3X63Ag8U.1 mouse myeloma cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL Life Technologies, NY) supplemented with 10% fetal bovine serum as described.[16] To assess viable cell numbers, we used the Dojindo Cell Counting Kit-8 (CCK8 kit, Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions.

The 293T, Huh-7, and U-2 OS cells were transfected with plasmid DNA by using the calcium phosphate method or FuGENE 6 Transfection Reagent (Roche Diagnostics, Mannheim, Germany) as described.[16] Short interfering RNA (siRNA) were transfected at a final concentration of 25 nM by using siPORT NeoFX Transfection Agent (Ambion, Austin, TX) following the manufacturer's instructions. Twenty-four hours after transfection, the medium was replaced with fresh medium containing fetal bovine serum, and the culture was continued for another 24 or 48 hours. Then, the cells were harvested for analysis. All transfection assays were repeated at least 3 times.

Plasmids and siRNA. Human wild-type gankyrin cDNAs, full coding sequence and deletion mutants, were cloned into the mammalian expression vector pMKIT-NEO and expressed as hemagglutinin (HA)-tagged proteins (Fig. 1A). Full-length gankyrin was expressed without a tag as well. To obtain recombinant human gankyrin protein, the full-length cDNA was cloned into an expression vector derived from pET28 (Novagen, EMD Biosciences Inc., San Diego, CA) and expressed as hexahistidine-tagged protein.

To down-regulate gene expression, Silencer Predesigned siRNAs for gankyrin (Ambion) and Stealth Select iRNA: for IGFBP-5 (Invitrogen, Tokyo, Japan), were used together with respective control RNAs.

Antibodies. To obtain monoclonal antibodies against human gankyrin, recombinant (His)6-gankyrin protein was used as an immunogen. It was dissolved in phosphate-buffered saline (1 mg/mL) and emulsified with an equal volume of Freund's complete adjuvant (Difco, Becton-Dickinson, Franklin Lakes, NJ). Two female BALB/c mice were injected with the emulsion (50 μL/mouse) in the footpad. Two weeks after immunization, the inguinal lymph node cells (4×10^7 cells) were fused with P3X63Ag8U.1 myeloma cells (1×10^7) using polyethylene glycol 1500 (Roche Diagnostics). Fused cells were cultured in 96-well plates at 2×10^5 cell/well. The supernatants were assayed for the anti-gankyrin antibody titer by an enzyme-linked immunosorbent assay using recombinant His-tagged, glutathione-S-transferase (GST)-

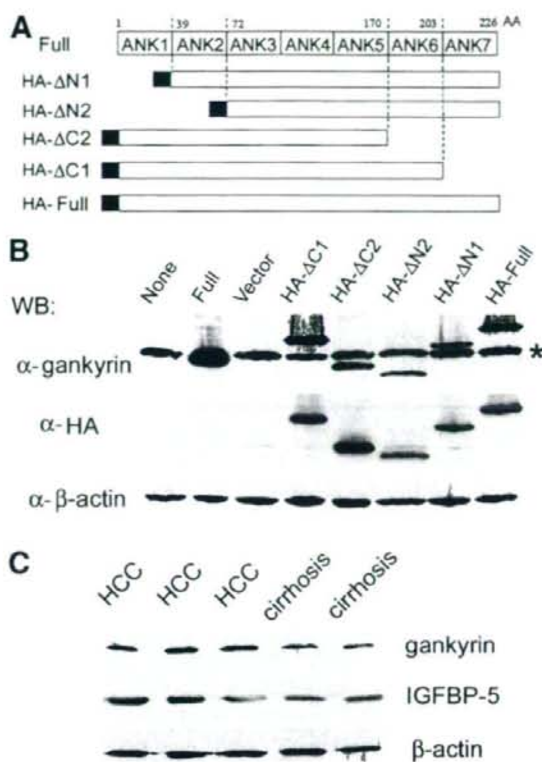

Fig. 1. Recognition of gankyrin protein by the monoclonal antibody. (A) Structures of wild-type gankyrin (Full) and its deletion mutants. Numbers on top, N- and C-terminal amino-acid residues. ANK, ankyrin repeat. Black bars, HA tags. (B) Specificity of the antibody. 293T cells were transfected with plasmids expressing the indicated proteins. Cell lysates were analyzed by western blotting (WB) using the anti-gankyrin monoclonal antibody (3A6C2), anti-HA antibody, and anti-β-actin antibody. *Mobility of the endogenous gankyrin. Representative results of 3 repeated experiments are shown. (C) Detection of gankyrin protein in tissues. Lysates were made from hepatocellular carcinoma (HCC, n = 3) and cirrhotic liver tissues (n = 2), and analyzed by WB using antibodies for indicated proteins. HA, hemagglutinin.

tagged, and nontagged gankyrin proteins. Selected relevant hybridomas were cloned by the limiting dilution method, and the isotypes of secreted monoclonal antibodies were determined by the IsoStrip kit (Roche Diagnostics) following the manufacturer's instructions. Finally, an IgG2b kappa monoclonal antibody that showed the highest affinity for gankyrin was obtained and named 3A6C2.

For western blot analysis, mouse monoclonal anti-gankyrin antibody (3A6C2), goat polyclonal anti–IGFBP-5 antibody (R&D Systems Inc., Minneapolis, MN), mouse monoclonal anti-HA antibody (12CA5, Roche Diagnostics), and mouse monoclonal anti–β-actin antibody (Chemicon International, Temecula, CA) were

used. Horseradish peroxidase–conjugated secondary antibodies against mouse or goat immunoglobulins were obtained from DAKO (Kyoto, Japan).

For immunohistochemistry, mouse monoclonal anti-gankyrin (3A6C2), anti-MDM2 (Ab-4, Oncogene research products, Boston, MA), and anti-p53 (DO-7, DAKO) antibodies, rabbit polyclonal anti-IGFBP-5 antibody (GroPep, Thebarton, Australia), and horseradish peroxidase–conjugated secondary antibodies against mouse or rabbit immunoglobulins (DAKO) were used.

Analysis of Gene Expression. Extraction of RNA, preparation of cell and tissue lysates, and western blot analysis were performed as described.[9] Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis was performed using ABI PRISM 7900 (Applied Biosystems, Foster City, CA) and a 1-step QuantiTect RT-PCR Kit (Qiagen, Cowley, UK) according to the manufacturer's instructions. PCR conditions were 50°C for 30 minutes and 95°C for 15 minutes, followed by 45 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 45 seconds. Specific PCR amplification products were detected by SYBR Green. Transcripts of β-actin were quantified as control. Primer sequences used were as follows: IGFBP-5, AAGAAGCTGACCCAGTCCAA and GAATCCTTTGCGGTCACAAT; gankyrin, GCAACTTGGAGTGCCAGTGAA and TCACTTGAGCACCTTTTCCCA; β-actin, CTACGTCGCCCTGGACTTCGAGC and GATGGAGCCGCCGATCCACACGG.

The immunohistochemical staining was performed on 4-μm-thick paraffin sections of tissues fixed in buffered formalin. The sections were pretreated with 10 mM citrate buffer (pH 6.1) in a microwave oven for 5 minutes. Endogenous peroxidase activity was blocked with 0.3 % H_2O_2 for 10 minutes. The sections were incubated with 10% fetal bovine serum for 30 minutes to reduce nonspecific binding, followed by incubation with the primary antibody at 4°C overnight. They were subsequently incubated with horseradish peroxidase–conjugated anti-mouse or rabbit immunoglobulin antibody for 30 minutes. The enzymatic reaction was developed in a freshly prepared solution of 3,3′-diaminobenzidine tetrahydrochloride using DAKO Liquid DAB Substrate-Chromogen Solution for 10 minutes at room temperature. The sections were then counterstained with hematoxylin. The staining pattern, the distribution of the immunostaining in each tissue, and the intensity of the staining were studied in detail. Negative controls were conducted by substituting normal sera of each animal for the primary antibodies. When immunoreactivities were heterogenously observed, cases with moderate or strong staining of nucleus or cytoplasm in more than 5% of the cells were considered positive. To analyze the correlation of the expression levels of gankyrin and IGFBP-5, the staining intensity was expressed as 0 (negative), 1+ (weakly positive), 2+ (moderately positive), or 3+ (strongly positive). In each case the immunoreactivity was determined in 5 random high-powered fields and the count was done independently by 2 observers.

Statistical Analysis. Categorical variables were compared using Fisher's exact test. Paired comparison of continuous data was performed using the Wilcoxon signed ranks test. To assess whether the 2 variables covary, Spearman's rank correlation coefficient was determined. Cumulative survival curves were calculated by the Kaplan-Meier method and analyzed by the log-rank test. All statistical analyses were performed using the JMP statistical software package (SAS Institute Inc., Cary, NC). A P value less than 0.05 was considered statistically significant.

Results

Clinicopathological Profiles. Forty-three patients with HCC were recruited in this study, including 27 men and 16 women, with ages ranging from 25 to 78 (median 65) years old. Clinicopathological profiles of the patients and their HCCs are shown in Table 1. Antibody to hepatitis C virus was found in sera of 72% of the patients, and hepatitis B virus surface antigen was positive in 21%.

According to the TNM staging, 60% were stage I to II and 40% were stage III to IV. In noncancerous portions of the resected livers, cirrhosis and chronic hepatitis[18] were found in 68% and 30%, respectively, of the specimens, whereas only 1 (2%) was of normal histology. Fibrocapsular formation surrounding HCC was observed in 84% and capsular invasion by HCC cells in 33%. Portal vein involvement and satellite nodules suggesting intrahepatic metastasis were found in 21% and 37%, respectively.

Detection of Gankyrin with the Monoclonal Anti-gankyrin Antibody. To determine the specificity of the monoclonal anti-gankyrin antibody 3A6C2, we expressed wild-type full-length or truncated gankyrin (Fig. 1A) in 293T cells. The antibody detected all mutants of gankyrin, suggesting that the epitope exists within the third and fifth ankyrin-repeat region (Fig. 1B). The antibody recognized the endogenous gankyrin as well, and no major cross-reacting band was observed.

Because gankyrin mRNA is known to be overexpressed in most HCCs,[9] we analyzed the levels of gankyrin protein in HCCs and surrounding noncancerous liver tissues using the 3A6C2 antibody. The protein level of gankyrin was higher in HCC tissues than in noncancerous tissues

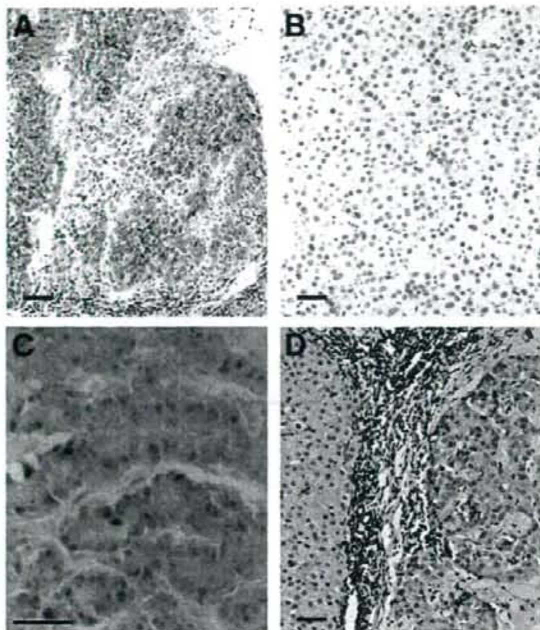

Fig. 2. Immunohistochemical detection of gankyrin in hepatocellular carcinoma (HCC). HCC sections were stained with mouse monoclonal anti-gankyrin antibody, and counterstained with hematoxylin. Positive immunostaining appears brown. (A) Positive staining for gankyrin in the cytoplasm of most HCC cells. (B) Barely detectable gankyrin signal in some HCC cells. (C) Presence of gankyrin in the nucleus of some HCC cells. (D) Stronger staining for gankyrin in HCC cells (right) than the neighboring cirrhotic hepatocytes (left). Bar, 50 μm.

(Fig. 1C). The mobilities of the gankyrin band were not different among samples.

Immunohistochemical Analysis of Gankyrin Expression. We next examined the expression of gankyrin protein in HCC and noncancerous liver tissues by immunohistochemistry. The gankyrin signal was observed mainly in the cytoplasm and occasionally in the nucleus of HCC cells (Fig. 2A-C). Although at lower levels compared with those in HCCs, weak but reproducible gankyrin signals were observed in the cytoplasm of the hepatocytes in the noncancerous tissues (Fig. 2D). Expression of gankyrin was not detected in the bile duct cells, blood endothelial cells, or other nonparenchymal cells in the liver tissues. Of 43 HCCs examined, the cytoplasm was stained positively for gankyrin in 27 (63%), and 9 of them (21%) were also positive for nuclear staining. Of 32 noncancerous liver tissues available, gankyrin was positive in 17 (53%).

As shown in Table 2, we analyzed an association between gankyrin protein expression and clinicopathological findings. No significant association between gankyrin expression in HCC cells and sex, age, tumor size, fibrotic change in noncancerous liver tissues, differentiation of the tumor cells, or hepatitis B or C virus infection was observed. Positive cytoplasmic staining for gankyrin of HCC cells was significantly associated with low TNM stage (stage I or II; $P = 0.004$), no capsular invasion ($P = 0.018$), no portal venous invasion ($P = 0.008$), and no intrahepatic metastasis ($P = 0.012$) of HCC. In noncancerous liver tissues, positive gankyrin staining of hepatocytes was associated with the cytoplasmic gankyrin positivity of HCC cells of the same patient ($P = 0.021$, Table 3), but not with the parameters examined except for the serum alpha-fetoprotein level ($P = 0.015$, Table 2).

Because expression of gankyrin affects the degradation of p53 and MDM2,[16] we examined the expression of p53 and MDM2 as well as gankyrin in HCCs. By immunohistochemistry, nuclear expression of p53 and MDM2 were detected in 30% and 23%, respectively, of 43 HCCs (Fig. 3, Table 3). Positive staining for gankyrin was not associated with the staining for p53 nor MDM2 in HCC cells.

Up-regulation of IGFBP-5 Expression by Gankyrin in HCCs. Preliminary microarray analysis of the cDNA libraries prepared from U-2 OS cells and Huh-7 cells overexpressing gankyrin suggested that IGFBP-5 mRNA was up-regulated by gankyrin (A. Umemura and J. Fujita, unpublished data). Real-time RT-PCR analysis con-

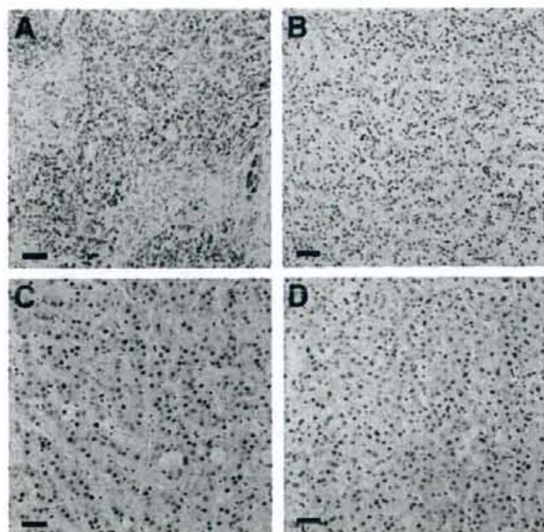

Fig. 3. Immunohistochemical detection of p53 and MDM2 in hepatocellular carcinoma (HCC). HCC sections were stained with antibodies specific to p53 (A and B) or MDM2 (C and D), and counterstained with hematoxylin. Positive immunostaining appears brown. (A) Positive staining for p53 in the nucleus of most HCC cells. (B) Negative p53 in HCC cells. (C) Positive staining for MDM2 in the nucleus of most HCC cells. (D) Negative MDM2 in HCC cells. Bar, 50 μm.

Table 2. Gankyrin Expression and Clinicopathological Characteristics

	Gankyrin Expression in the Cytoplasm of					
	HCC			Noncancerous Liver		
	Negative (n = 16)	Positive (n = 27)	*P* value	Negative (n = 15)	Positive (n = 17)	*P* value
Sex distribution						
Male	12	15	0.328	10	11	1.000
Female	4	12		5	6	
Median age (years)	64	65	0.696	63	62	0.649
Virus marker			NS			NS
HBV(+)/HCV(−)	3	3		2	2	
HBV(−)/HCV(+)	10	18		11	11	
HBV(+)/HCV(+)	1	2		2	0	
HBV(−)/HCV(−)	2	4		0	4	
Median AFP (ng/mL)	63.0	95.0	0.890	25.0	199.0	0.015
Median tumor size (cm)	4.5	4.0	0.098	4.5	4.0	0.372
Liver cirrhosis (+)	9	20	0.316	9	13	0.450
TNM stage						
I and II	5	21	0.004	8	12	0.467
III and IV	11	6		7	5	
Histological differentiation						
Well	5	7	0.737	6	3	0.243
Moderate and poor	11	20		9	14	
Capsular formation (+)	15	21	0.229	12	13	1.000
Capsular invasion (+)	9	5	0.018	4	6	0.712
Portal venous invasion (+)	7	2	0.008	4	3	0.678
Intrahepatic metastasis (+)	10	6	0.012	6	5	0.712
Gankyrin nuclear expression						
Yes	0	9	0.016	2	5	0.403
No	16	18		13	12	

Abbreviations: HCV, anti-hepatitis C virus antibody; HBV, hepatitis B surface antigen; (+), positive or present; (−), negative or absent; AFP, serum alpha-fetoprotein; NS, not significant between any groups or combinations thereof.

firmed that overexpression of gankyrin increased the IGFBP-5 mRNA levels 5.2-fold and 1.7-fold (mean, n = 3 each) in U-2 OS and Huh-7 cells, respectively, and western blot analysis demonstrated that the protein levels were increased as well (Fig. 4A). Conversely, when gankyrin expression was suppressed by siRNA, IGFBP-5 expression was down-regulated (Fig. 4B). In 2 of 3 HCC tissues overexpressing gankyrin, the levels of IGFBP-5 protein were higher compared with those in noncancerous tissues (Fig. 1C). To identify a role that IGFBP-5 might play in HCC cells, we next suppressed IGFBP-5 expression by siRNA. No apoptosis was induced, but viable cell numbers were decreased in Huh-7 as well as U-2 OS cells (Fig. 4C,D, and data not shown), suggesting a growth-promoting effect of IGFBP-5.

The expression of IGFBP-5 was further examined immunohistochemically in 43 HCC and 32 noncancerous liver tissues (Fig. 5, Table 3). In 42% of HCCs, IGFBP-5 was positively stained in the cytoplasm of HCC cells (Fig. 5A). IGFBP-5 was also detected, although at lower levels, in the cytoplasm of hepatocytes in 28% of the noncancerous tissues (Fig. 5B-D), but not in bile duct cells, blood endothelial cells, or other nonparenchymal cells.

Specific cytoplasmic staining for IGFBP-5 in HCC cells was associated with low TNM stage (stage I or II; $P = 0.013$), no portal venous invasion ($P = 0.006$), low serum alpha-fetoprotein value ($P = 0.031$), and small tumor size ($P = 0.009$). No association with capsular invasion or intrahepatic metastasis was observed. There was a significant association between positivities for IGFBP-5 and

Table 3. Gankyrin Expression and Molecular Histological Markers

	Gankyrin Expression in HCC		
	Negative	Positive	*P* value
Gankyrin expression in non-HCC			
Negative (n = 15)	8	7	0.021
Positive (n = 17)	2	15	
p53 expression in HCC			
Negative (n = 30)	11	19	1.000
Positive (n = 13)	5	8	
MDM2 expression in HCC			
Negative (n = 33)	14	19	0.276
Positive (n = 10)	2	8	
IGFBP-5 expression in HCC			
Negative (n = 25)	13	12	0.026
Positive (n = 18)	3	15	
IGFBP-5 expression in non-HCC			
Negative (n = 23)	14	9	0.011
Positive (n = 9)	1	8	

Abbreviations: HCC, hepatocellular carcinoma; non-HCC, noncancerous portion of the resected liver; IGFBP-5, insulin-like growth factor-binding protein 5.

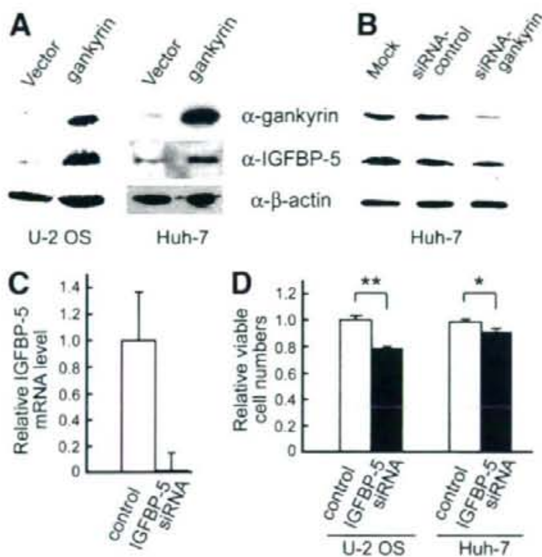

Fig. 4. Induction of IGFBP-5 by gankyrin. (A) U-2 OS cells (lanes 1 and 2) and Huh-7 cells (lanes 3 and 4) transiently transfected with plasmids expressing gankyrin or vector alone were analyzed for expression of IGFBP-5 by western blotting using the indicated antibodies. Representative results from more than 3 experiments are shown. (B) Huh-7 cells, mock transfected or transfected with siRNA for gankyrin or control RNA as indicated, were analyzed as in (A). (C) Suppression of IGFBP-5 expression by siRNA. Huh-7 cells were transfected with control RNA or IGFBP-5-specific siRNA. IGFBP-5 transcript levels were determined by real-time RT-PCR and normalized with β-actin levels. Results from 3 repeats were averaged and expressed relative to control. Error bars refer to standard deviation of the average quantitated results. (D) Effect of IGFBP-5 down-regulation on cell growth. U-2 OS and Huh-7 cells were transfected with IGFBP-5 siRNA or control RNA, and 72 hours later viable cell numbers were determined. Values are mean ± standard deviation ($n = 3$) and expressed relative to controls. ** and *, $P < 0.01$ and $P < 0.05$, respectively.

gankyrin (Table 3), and the levels of expression covaried both in HCCs ($\rho = 0.629$, $P < 0.001$) (Fig. 5E) and noncancerous hepatocytes ($\rho = 0.606$, $P < 0.001$) (Fig. 5F).

Expression of Gankyrin in HCC and Patient Prognosis. When we examined the relationship between gankyrin expression in HCC cells and the survival of patients after surgical resection, a significant difference was observed between the patients with gankyrin-positive HCCs and those with gankyrin-negative HCCs (Fig. 6). We found no significant difference in the survival rates between the patients whose HCCs stained positively and negatively for p53, MDM2, or IGFBP-5.

Discussion

Gankyrin is as an oncogene, mRNA of which is overexpressed in almost all human HCCs.[9,19] Although less frequent, gankyrin has been found by RNA dot blot analysis to be overexpressed in additional tumors including those of the breast, colon, rectum, stomach, small intestine, pancreas, ovary, lung, and thyroid (A. Umemura and J. Fujita, unpublished data). In the current study, we immunohistochemically examined the gankyrin protein expression in HCCs using the monoclonal anti-gankyrin antibody and found that the protein was highly expressed in the cytoplasm of 63% of HCCs. Tan et al.[20] has simi-

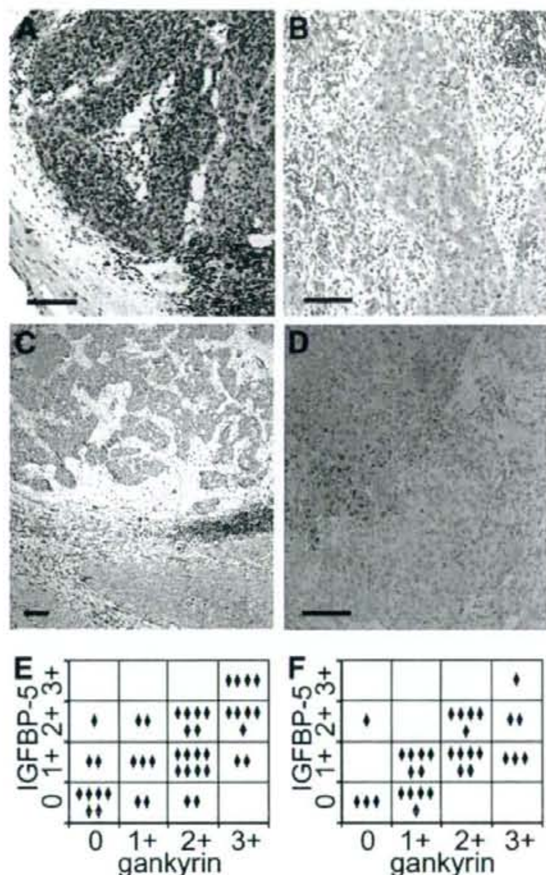

Fig. 5. Immunohistochemical detection of IGFBP-5 in hepatocellular carcinoma (HCC). HCC sections were stained with anti–IGFBP-5 antibody and counterstained with hematoxylin. Positive immunostaining appears brown. (A) Positive staining for IGFBP-5 in the cytoplasm of HCC cells, especially at the invasive boundaries. (B) Presence of IGFBP-5 in noncancerous cirrhotic hepatocytes. (C) Stronger staining for IGFBP-5 in HCC cells (upper) than the neighboring cirrhotic hepatocytes (lower). (D) Positive staining for IGFBP-5 in HCC cells (upper left), but negative in cirrhotic cells (lower right). Bar, 100 μm. (E) Correlation of expression levels of gankyrin and IGFBP-5 in HCCs. The immunostaining levels were expressed as 0 (negative), 1+ (weakly positive), 2+ (moderately positive), or 3+ (strongly positive). Each diamond represents 1 case. The Spearman's rho = 0.629, $P < 0.001$. (F) Correlation of expression levels of gankyrin and IGFBP-5 in noncancerous hepatocytes determined as in (E). The Spearman's rho = 0.606, $P < 0.001$.

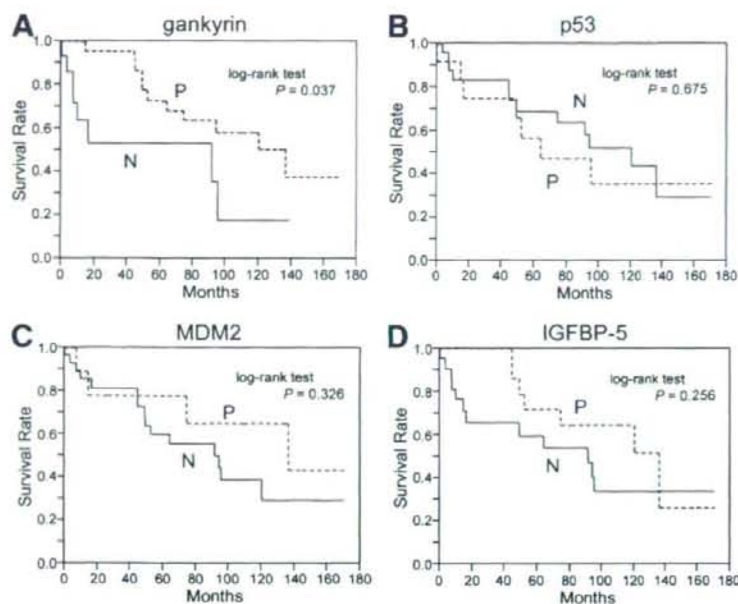

Fig. 6. Survival of patients and expression of molecular markers. The Kaplan-Meier method was used to determine the patient survival and log-rank test to compare survival between patients with HCC grouped according to (A) gankyrin positivity, (B) p53 positivity, (C) MDM2 positivity, and (D) IGFBP-5 positivity. P, positive. N, negative.

larly found overexpression of gankyrin protein in 60% of HCCs using a polyclonal antibody. The reason why the protein is not overexpressed in one-third of HCCs despite overexpression of its mRNA is unknown. The posttranscriptional, translational, and posttranslational regulations of gankyrin expression remain to be elucidated.

According to the 15th follow-up survey by the Liver Cancer Study group of Japan, the cumulative survival rates after surgical removal of HCC are 52.3% and 27.3% at 5 and 10 years, respectively, and better survival rates are associated with fewer numbers of tumors, lack of portal venous invasion, and early clinical stages.[4-6] Consistent with these observations, gankyrin positivity of HCC was associated with low TNM stage, lack of capsular invasion, portal venous invasion, and intrahepatic metastasis, and better prognosis of the patients. Patients with hyperdiploid acute lymphoblastic leukemia with more than 50 chromosomes, one of the 6 subtypes of pediatric acute lymphoblastic leukemia, have an excellent prognosis compared with other subtypes, and interestingly, overexpression of gankyrin is 1 of the diagnostic and subclassification markers for it.[21] Expression of gankyrin protein may be used as a marker for better prognosis of the patients with HCC as well.

The gankyrin oncoprotein plays a key role in regulation of cell cycle and apoptosis, at least in cultured cells, by inhibiting Rb and p53.[10] In a rodent hepatocarcinogenesis model, hypermethylation of the p16INK4A gene and p53 mutation appear at a late stage, whereas gankyrin is overexpressed from early after carcinogen treatment, preceding the loss of Rb protein and adenoma formation.[22] Clinically, p53 mutation is not so frequent in HCCs (15%-30%), especially in low-grade or low-stage HCCs.[23,24] Tan et al.[20] have immunohistochemically detected gankyrin overexpression in 82%, 63%, and 22% of Edmondson's grade I to II, III, and IV HCCs, respectively. We observed gankyrin positivity in 81% and 35% of low and high TNM stage HCCs, respectively. These results suggest that gankyrin plays an important role(s) at early stages of hepatocarcinogenesis by suppressing Rb, p53 and possibly other tumor suppressors. In advanced HCCs, by contrast, oncogenic mutations probably have accumulated in many genes including p53, and overexpression of gankyrin may not be so crucial as in early stage HCCs. This could explain the present association of gankyrin-negative HCCs with poorer prognosis and the finding that both cases of gankyrin-negative HCCs with gankyrin-positive noncancerous hepatocytes belonged to high TNM stages. This is, however, one of several possible explanations, and further work is necessary to clarify the exact reasons for the observed association.

By immunohistochemical staining, p53 has been detected in 20% to 30% of HCCs.[25, 26] Although strong immunohistochemical reactivity for p53 may not be an indicator of the presence of p53 gene mutations as initially suggested,[26] it has been associated in some studies with higher proliferative activity, lower differentiation of HCC cells, or poorer survival of patients. Endo et al.[27] immunohistochemically detected MDM2 in 28 of 107 (26%) HCCs, and the positive expression correlated with

the presence of p53 mutation and poorer prognosis, although it also correlated with smaller HCC size and the absence of vascular invasion. We immunohistochemically detected the expression of p53 and MDM2 in 30% and 23%, respectively, of HCCs, which is in accord with other studies, but no correlation was seen between expression and survival of the patients. Gankyrin accelerates degradation of Rb, p53, and MDM2 in cultured cells.[9,16] Although some correlation between expression of gankyrin and Rb has been suggested in HCC tissues,[20] we did not observe significant relationship between the gankyrin positivity and negative staining for p53 nor MDM2. The analysis of individual cells for protein expression, for example by double 2-color immunostaining, may have revealed the presence of some relationship. But most probably, our finding reflects complex interrelated mechanisms regulating the levels of these proteins and also suggests that the relevance of the effects of gankyrin on p53, MDM2, and Rb demonstrated in cultured cells to human hepatocarcinogenic process remains to be firmly established.

The 6 members of IGFBP family (IGFBP-1 through IGFBP-6) are important components of the insulin-like growth factor (IGF) axis, and regulate the activity of both IGF-I and IGF-II polypeptide growth factors.[28] IGF-I, IGF-II, and their receptors are expressed in a wide variety of cells, and the liver is the main source of circulating IGF-I. IGFBPs are also secreted by many cell types, and their expression is regulated in a cell-dependent and tissue-type–dependent manner. In the current study, we found up-regulation of IGFBP-5 mRNA and protein levels by overexpression of gankyrin in human osteosarcoma and HCC cell lines and consistently detected a significant association between the protein levels of gankyrin and IGFBP-5 in HCC specimens. In the proximal promoter region of the IGFBP-5 gene, there are several putative transcription-factor–binding sites including those for AP-2, c-Myb, C/EBP, and NF-1, and responsive elements to prostaglandin E_2, cyclic adenosine monophosphate, progesterone/retinoic acid, and Akt.[28] Whether the effect of gankyrin on IGFBP-5 expression is mediated by these factors is unknown.

The IGFBPs bind IGFs with high affinity, and they are able to enhance or inhibit the activity of IGFs in a cell-specific and tissue-type–specific manner.[28] In addition, IGFBPs have IGF-independent effects. There are several reports on the relationship between the IGF axis and HCC.[29-31] IGFBP-3 is the most abundant IGFBP present in noncancerous liver tissue and could serve as a negative regulator of cell proliferation in human HCCs.[32] Although the presence of IGFBP-5 in numerous tumors and cell lines has been demonstrated, its expression and significance in human HCC have not been documented. We found positive staining for IGFBP-5 in 42% of HCCs, and the positivity correlated with absence of portal venous invasion, low TNM stage, and small tumor size. Although not statistically significant, patients with IGFBP-5–positive HCCs tended to survive longer than those with IGFBP-5–negative HCCs. These findings are essentially similar to those observed for gankyrin. Regarding the effect of IGFBP-5 on cell proliferation, there are contradictory findings.[28] In breast cancer cells, many studies have reported inhibition of growth, but there are some indicating a stimulatory effect.[33] IGFBP-5 is up-regulated in involuting prostate but is also implicated in growth stimulation of prostate tumor cells.[34] We found that down-regulation of IGFBP-5 suppresses growth of Huh-7 HCC cells. Thus, these findings are consistent with a notion that high expression of IGFBP-5 and gankyrin play oncogenic roles in HCCs of early clinical stages. Clarification of the exact roles played by them will shed more light on the molecular mechanisms of human hepatocarcinogenesis and lead to development of new therapeutic and preventive strategies.

Acknowledgment: We thank Dr. R. John Mayer for helpful suggestions.

References

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74-108.
2. Thomas MB, Zhu AX. Hepatocellular carcinoma: the need for progress. J Clin Oncol 2005;23:2892-2899.
3. Treiber G, Wex T, Rocken C, Fostitsch P, Malfertheiner P. Impact of biomarkers on disease survival and progression in patients treated with octreotide for advanced hepatocellular carcinoma. J Cancer Res Clin Oncol 2006;132:699-708.
4. Shimada K, Sano T, Sakamoto Y, Kosuge T. A long-term follow-up and management study of hepatocellular carcinoma patients surviving for 10 years or longer after curative hepatectomy. Cancer 2005;104:1939-1947.
5. Poon RT, Fan ST, Ng IO, Lo CM, Liu CL, Wong J. Different risk factors and prognosis for early and late intrahepatic recurrence after resection of hepatocellular carcinoma. Cancer 2000;89:500-507.
6. Kiyosawa K, Umemura T, Ichijo T, Matsumoto A, Yoshizawa K, Gad A, et al. Hepatocellular carcinoma: recent trends in Japan. Gastroenterology 2004;127(5 Suppl 1):S17-S26.
7. Higashitsuji H, Higashitsuji H, Nagao T, Nonoguchi K, Fujii S, Itoh K, et al. A novel protein overexpressed in hepatoma accelerates export of NF-kappa B from the nucleus and inhibits p53-dependent apoptosis. Cancer Cell 2002;2:335-346.
8. Gotoh K, Nonoguchi K, Higashitsuji H, Kaneko Y, Sakurai T, Sumitomo Y, et al. Apg-2 has a chaperone-like activity similar to Hsp110 and is overexpressed in hepatocellular carcinomas. FEBS Lett 2004;560:19-24.
9. Higashitsuji H, Itoh K, Nagao T, Dawson S, Nonoguchi K, Kido T, et al. Reduced stability of retinoblastoma protein by gankyrin, an oncogenic ankyrin-repeat protein overexpressed in hepatomas. Nat Med 2000;6:96-99.
10. Higashitsuji H, Liu Y, Mayer RJ, Fujita J. The oncoprotein gankyrin negatively regulates both p53 and RB by enhancing proteasomal degradation. Cell Cycle 2005;4:1335-1337.
11. Hori T, Kato S, Saeki M, DeMartino GN, Slaughter CA, Takeuchi J, et al. cDNA cloning and functional analysis of p28 (Nas6p) and p40.5 (Nas7p),

two novel regulatory subunits of the 26S proteasome. Gene 1998;216:113-122.

12. Dawson S, Apcher S, Mee M, Higashitsuji H, Baker R, Uhle S, et al. Gankyrin is an ankyrin-repeat oncoprotein that interacts with CDK4 kinase and the S6 ATPase of the 26 S proteasome. J Biol Chem 2002;277:10893-10902.
13. Li J, Tsai MD. Novel insights into the INK4-CDK4/6-Rb pathway: counter action of gankyrin against INK4 proteins regulates the CDK4-mediated phosphorylation of Rb. Biochemistry 2002;41:3977-3983.
14. Shan YF, Zhou WP, Fu XY, Yan HX, Yang W, Liu SQ, et al. The role of p28GANK in rat oval cells activation and proliferation. Liver Int 2006;26:240-247.
15. Iwai A, Marusawa H, Kiuchi T, Higashitsuji H, Tanaka K, Fujita J, et al. Role of a novel oncogenic protein, gankyrin, in hepatocyte proliferation. J Gastroenterol 2003;38:751-758.
16. Higashitsuji H, Higashitsuji H, Itoh K, Sakurai T, Nagao T, Sumitomo Y, et al. The oncoprotein gankyrin binds to MDM2/HDM2, enhancing ubiquitylation and degradation of p53. Cancer Cell 2005;8:75-87.
17. Ueno S, Tanabe G, Nuruki K, Hamanoue M, Komorizono Y, Oketani M, et al. Prognostic performance of the new classification of primary liver cancer of Japan (4th edition) for patients with hepatocellular carcinoma: a validation analysis. Hepatol Res 2002;24:395-403.
18. Desmet VJ. Histological classification of chronic hepatitis. Acta Gastroenterol Belg 1997;60:259-267.
19. Fu XY, Wang HY, Tan L, Liu SQ, Cao HF, Wu MC. Overexpression of p28/gankyrin in human hepatocellular carcinoma and its clinical significance. World J Gastroenterol 2002;8:638-643.
20. Tan L, Fu XY, Liu SQ, Li HH, Hong Y, Wu MC, et al. Expression of p28GANK and its correlation with RB in human hepatocellular carcinoma. Liver Int 2005;25:667-676.
21. Yeoh EJ, Ross ME, Shurtleff SA, Williams WK, Patel D, Mahfouz R, et al. Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. Cancer Cell 2002;1:133-143.
22. Park TJ, Kim HS, Byun KH, Jang JJ, Lee YS, Lim IK. Sequential changes in hepatocarcinogenesis induced by diethylnitrosamine plus thioacetamide in Fischer 344 rats: induction of gankyrin expression in liver fibrosis, pRB degradation in cirrhosis, and methylation of p16(INK4A) exon 1 in hepatocellular carcinoma. Mol Carcinog 2001;30:138-150.
23. Tanaka S, Toh Y, Adachi E, Matsumata T, Mori R, Sugimachi K. Tumor progression in hepatocellular carcinoma may be mediated by p53 mutation. Cancer Res 1993;53:2884-2887.
24. Hayashi H, Sugio K, Matsumata T, Adachi E, Takenaka K, Sugimachi K. The clinical significance of p53 gene mutation in hepatocellular carcinomas from Japan. HEPATOLOGY 1995;22:1702-1707.
25. Nagao T, Kondo F, Sato T, Nagato Y, Kondo Y. Immunohistochemical detection of aberrant p53 expression in hepatocellular carcinoma: correlation with cell proliferative activity indices, including mitotic index and MIB-1 immunostaining. Hum Pathol 1995;26:326-333.
26. Anzola M, Saiz A, Cuevas N, Lopez-Martinez M, Martinez de Pancorbo MA, Burgos JJ, et al. High levels of p53 protein expression do not correlate with p53 mutations in hepatocellular carcinoma. J Viral Hepat 2004;11:502-510.
27. Endo K, Ueda T, Ohta T, Terada T. Protein expression of MDM2 and its clinicopathological relationships in human hepatocellular carcinoma. Liver 2000;20:209-215.
28. Beattie J, Allan GJ, Lochrie JD, Flint DJ. Insulin-like growth factor-binding protein-5 (IGFBP-5): a critical member of the IGF axis. Biochem J 2006;395:1-19.
29. Gong Y, Cui L, Minuk GY. The expression of insulin-like growth factor binding proteins in human hepatocellular carcinoma. Mol Cell Biochem 2000;207:101-104.
30. Scharf JG, Dombrowski F, Ramadori G. The IGF axis and hepatocarcinogenesis. Mol Pathol 2001;54:138-144.
31. Breuhahn K, Longerich T, Schirmacher P. Dysregulation of growth factor signaling in human hepatocellular carcinoma. Oncogene 2006;25:3787-3800.
32. Huynh H, Chow PK, Ooi LL, Soo KC. A possible role for insulin-like growth factor-binding protein-3 autocrine/paracrine loops in controlling hepatocellular carcinoma cell proliferation. Cell Growth Differ 2002;13:115-122.
33. McCaig C, Perks CM, Holly JM. Intrinsic actions of IGFBP-3 and IGFBP-5 on Hs578T breast cancer epithelial cells: inhibition or accentuation of attachment and survival is dependent upon the presence of fibronectin. J Cell Sci 2002;115:4293-4303.
34. Miyake H, Pollak M, Gleave ME. Castration-induced up-regulation of insulin-like growth factor binding protein-5 potentiates insulin-like growth factor-I activity and accelerates progression to androgen independence in prostate cancer models. Cancer Res 2000;60:3058-3064.

Hepatology Research 2008; 38: 27–36 doi: 10.1111/j.1872-034X.2007.00217.x

Special Report

Guidelines for the antiviral therapy of hepatitis C virus carriers with normal serum aminotransferase based on platelet counts

Takeshi Okanoue,[1] Yoshito Itoh,[1] Masahito Minami,[1] Hiroaki Hashimoto,[1] Kohichiro Yasui,[1] Hiroshi Yotsuyanagi,[2] Tetsuo Takehara,[3] Takashi Kumada,[4] Eiji Tanaka,[5] Shuhei Nishiguchi,[6] Namiki Izumi,[7] Michio Sata,[8] Morikazu Onji,[9] Gotaro Yamada,[10] Kiwamu Okita[11] and Hiromitsu Kumada[12]

[1]*Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Kyoto,* [2]*Department of Infectious Diseases, University of Tokyo, Tokyo,* [3]*Department of Gastroenterology and Hepatology, Osaka University, Osaka,* [4]*Department of Gastroenterology, Ogaki Municipal Hospital, Gifu,* [5]*Department of Internal Medicine, Shinshu University, Matsumoto,* [6]*Department of Internal Medicine, Hyogo College of Medicine, Hyogo,* [7]*Department of Gastroenterology and Hepatology, Musashino Red-Cross Hospital, Musashino,* [8]*Second Department of Internal Medicine, Kurume University, Kurume,* [9]*Department of Gastroenterology and Metabology, Ehime University, Matsuyama,* [10]*Department of Gastroenterology and Metabology, Kawasaki Hospital, Okayama,* [11]*Center of Liver Disease, Social Insurance Alliance Shimonoseki Hospital, and* [12]*Department of Hepatology, Toranomon Hospital, Tokyo, Japan*

Aim: We aimed to identify the candidates for antiviral therapy, among patients who are hepatitis C virus (HCV) carriers with normal serum aminotransferase (ALT), focused on the inhibition of hepatocellular carcinoma (HCC).

Methods: Four hundred and sixty-four HCV carriers with normal serum ALT and 129 HCV carriers with persistently normal ALT (PNALT) and platelet (PLT) counts ≥150 000/μL who received liver biopsies were enrolled. HCV carriers with normal serum ALT were divided into four groups according to their ALT levels (≤30 U/L or 31–40 U/L) and PLT counts (≥150 000/μL or <150 000/μL).

Results: In 129 HCV carriers with PNALT, the rate of progression of fibrosis stage was 0.05/year and no HCC was detected during the follow up for 10 years. Approximately 20% of patients with ALT ≤40 U/L and PLT counts ≥150 000/μL were at stage F2–3; however, approximately 50% of patients with ALT ≤ 40 U/L and PLT counts <150 000/μL were at stage F2–4. An algorithm for the management of HCV carriers with normal serum ALT was advocated based on ALT and PLT counts.

Conclusion: The combination of ALT and PLT counts is useful for evaluating the fibrosis stage in HCV carriers with normal serum ALT. Most patients with PLT counts <150 000/μL are candidates for antiviral therapy, especially those with ALT levels ≥31 U/L when we focus on the inhibition of the development of HCC.

Key words: antiviral therapy, chronic hepatitis C, hepatitis C virus carriers, normal serum aminotransferase, platelet count

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) caused by hepatitis C virus (HCV) infection usually develops in patients with advanced chronic hepatitis (CH) or liver cirrhosis. The antiviral treatment for chronic hepatitis C (CH-C) is useful for inhibiting hepatic inflammation and progression of hepatic fibrosis, and consequently the development of HCC.[1-6]

Serum aminotransferase (ALT) levels are within the normal ranges in 20–40% of patients with chronic HCV infection,[7-11] defining the upper limit of normal serum ALT as ≤40 U/L. Significant hepatic fibrosis (≥F2 by the METAVIR classification) has been demonstrated in 5–30% of such patients.[9,12-16] We reported previously

Correspondence: Dr Yoshito Itoh, Molecular Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-8566, Japan. Email: yitoh@koto.kpu-m.ac.jp
Received 6 March 2007; revision 22 May 2007; accepted 14 June 2007.

© 2007 The Japan Society of Hepatology

that HCV carriers with persistently normal ALT (PNALT) had histological features ranging from normal to minimal CH[17,18]; they showed slow progression of liver fibrosis and were at very low risk of developing HCC.[18]

The National Institute of Health Consensus Development Conference reported that HCV carriers with normal serum ALT are candidates for antiviral therapy.[19] A controlled study for the treatment of HCV carriers with PNALT with pegylated interferon alpha and ribavirin (PEG-IFN/Riba) for 48 weeks led to the eradication of HCV RNA in 40% of patients with genotype 1 and high viral load,[20] which is similar to the results of CH-C patients with elevated ALT levels.[21,22] However, it remains controversial whether these patients are candidates for antiviral therapy because of the limited efficacy of treatment, post-treatment flare-up, various side-effects, high cost of treatment, and their good prognoses.

In many Western countries, the upper limits of normal serum ALT are below 40 U/L;[23] however, a recent report from Italy demonstrated that the upper limit in healthy individuals was less than 30 U/L for men and 19 U/L for women.[24] We attempted to draft therapeutic guidelines for the treatment of HCV carriers with normal serum ALT. The biochemical and histological analyses were performed in HCV carriers with serum ALT levels below 40 U/L. These patients were divided into two groups based on ALT levels and then further divided into two subgroups according to their platelet (PLT) counts. We proposed an algorithm for the treatment of HCV carriers with normal serum ALT, taking into consideration the risk of progression to cirrhosis and the development of HCC. The present study demonstrated that the ranges of serum ALT and PLT counts are useful for deciding the indication of antiviral therapy for HCV carriers with normal serum ALT.

METHODS

Eligibility and definition

TWELVE HEPATOLOGISTS BELONGING to the Japanese Study Group of the Standard Antiviral Therapy for Viral Hepatitis, supported by the Ministry of Health, Labour and Welfare of Japan, which was settled on April 2004, participated in the study. Hiromitsu Kumada (Toranomon Hospital, Tokyo, Japan) serves as a chief and Takeshi Okanoue served as a researcher responsible for drafting the guidelines for the treatment of HCV carriers with normal serum ALT. In the present study, we tentatively defined the upper limit of the normal serum ALT as ≤40 U/L.

Patients with hepatitis B virus surface antigen, previous IFN treatment, history of heavy alcohol abuse, antinuclear antibody or antismooth muscle antibody, overt diabetes mellitus, or obesity (body mass index; ≥25 kg/m^2) were excluded from the study.

All of the patients underwent liver biopsy (≥2.0 cm in length) within 6 months prior to antiviral therapy, at which time their serum ALT levels were ≤40 U/L. Informed consent was obtained from every patient prior to liver biopsy and antiviral therapy.

Another study was conducted from January 1990 to August 2004 at Kyoto Prefectural University of Medicine (Kyoto, Japan). HCV carriers with PNALT were defined by serum ALT levels ≤30 U/L on at least three different occasions over a 12-month period and PLT counts ≥150 000/μL as reported previously.[18]

Study design

Among the 580 HCV carriers with normal serum ALT (≤40 U/L), 116 patients were excluded from the study because of insufficient data. Thus, 464 patients who received antiviral therapy from 1995 to 2004 were enrolled in this study (Table 1). Formalin-fixed liver specimens were stained with hematoxylin–eosin, and with Masson's trichrome. The liver specimens ($n = 262$) were also stained with Perls' Prussian blue to study hepatic iron loading. The histological findings were scored according to the classification proposed by Desmet *et al.*[25] and Ishak *et al.*[26] Steatosis was defined as fat droplets in >10% of hepatocytes. The degree of iron loading was assessed using a Perls' score of 0–4+, based on the scoring system of MacSween *et al.*[27]

The serum ALT, blood glucose level, immunoreactive insulin (IRI), serum ferritin, PLT count, serum hyaluronic acid, amount of serum HCV RNA, and the HCV genotype were examined. The homeostasis model assessment–insulin resistance was calculated as follows: plasma fasting glucose (mg/dL) × IRI (ng/mL) ÷ 405. The serum HCV RNA levels were determined using an Amplicor GT HCV monitor (Roche Diagnostic Systems, Tokyo, Japan). HCV genotype 1 (G1) and 2 (G2) were determined by a serologic genotyping assay.[28] G1 and G2 in this assay correspond to genotype 1 (1a, 1b) and 2 (2a, 2b) proposed by Simmonds *et al.*[29]

All the patients received IFN monotherapy or IFN/Riba combination therapy for 12–36 weeks. The average

© 2007 The Japan Society of Hepatology

Table 1 Baseline of hepatitis C virus patients with normal serum aminotransferase (ALT) received antiviral therapy

	ALT ≤ 30 U/L (group A)	ALT 31–40 U/L (group B)	*P*-value
No. patients	255	209	
Age	51.6 ± 13.0	53.5 ± 13.2	0.548*
Sex (male/female)	112/143	117/92	0.01**
BMI (kg/m^2)	21.6 ± 2.9	22.8 ± 3.0	<0.001*
HOMA-IR	2.5 ± 3.2	5.2 ± 6.5	0.093*
Genotype: 1/2/others	127/127/1	112/96/1	0.881**
Viral load: low/high	138/117	99/110	0.203**
G1 (low/high)	114/125		
G2 (low/high)	161/62		
Histology			
F stage (0/1/2/3/4)	29/166/48/11/1	22/122/57/6/2	0.169**
Grade (0/1/2/3)	25/187/41/2	7/159/43/0	0.046**
Fatty change† 0–1/2–4	232/23	161/48	0.033**
Iron load‡ 0/1–4	101/15	97/19	0.458**
Ferritin (ng/mL)	83.9 ± 103.7	118.8 ± 135.3	0.006*
PLT count (/μL)	19.2 ± 5.4	18.4 ± 6.1	0.059*
≥150 000/<150 000	204/51	141/68	0.002**
Hyaluronate (ng/mL)	60.8 ± 73.7	69.1 ± 73.0	0.249*
Duration of antiviral therapy (weeks)	25.6 ± 12.0	26.1 ± 12.1	0.297*
Effects of therapy			
SVR/non-SVR	142/113	99/110	0.075**

**P*-values were calculated by Mann–Whitney-*U*-test. **Fisher-exact-test. †0: no fatty change, 1: ≤10%, 2: 11–33%, 3: 34–66%, 4: ≥67% of hepatocyte; ‡no stain by 400×, 1: few stains by 250×, 2: stains by 100×, 3: stains by 25×, 4: stains by 10×. There were significant differences in sex distribution ($P = 0.01$), BMI ($P = 0.01$), frequency of steatosis ($P = 0.033$), serum ferritin level ($P = 0.006$), grade of hepatic inflammation ($P = 0.046$), incidence of fatty change ($P = 0.033$), serum ferritin level ($P = 0.006$), and the incidence of low PLT counts ($P = 0.002$) between groups A and B. Values are expressed as mean ± SD.
ALT, alanine aminotransferase; BMI, body mass index; HOMA-IR, homeostasis model assessment-insulin resistance; PLT, platelet; SVR, sustained viral responders.

duration of therapy between 1995 and 2003 was 26 weeks for IFN monotherapy and 24 weeks for IFN/Riba combination therapy. In principle, 6–10 MU IFN was administered daily for 2 weeks and three times per week subsequently. The daily dosage of ribavirin was 600–1000 mg depending on body weight. Sustained viral responders (SVR) were defined as patients who were negative for serum HCV RNA 6 months after the completion of antiviral therapy.

All of the patients were divided into two groups (group A: ALT ≤ 30 U/L, group B: 31 U/L ≤ ALT ≤ 40 U/L) which were further divided into two subgroups based on PLT counts: group A-1 and B-1 (PLT counts ≥150 000/μL) and groups A-2 and B-2 (PLT counts <150 000/μL).

One hundred and twenty-nine HCV carriers with PNALT were enrolled to determine their long-term prognosis. These patients showed normal serum ALT levels (≤30 U/L) over a 12-month period on least three different occasions (PLT counts ≥150 000/μL, and body mass index [BMI] <25 kg/m^2). Thirty-nine patients received serial liver biopsies. The mean follow-up period of the 129 patients was 7.2 ± 3.2 years on 15 November 2006.

Statistical analyses

Data are expressed as mean ± SD. We compared continuous variables using the Mann–Whitney *U*-test. A frequency analysis and comparison between the groups were performed using the χ^2-test or Fisher's exact test and the Mann–Whitney *U*-test. ANOVA and Tukey's HSD procedure was used to determine the difference between multiple groups. All tests were two-tailed and *P*-values of less than 0.05 were considered significant. All statistical analyses were performed using Statistical Package of Services Solutions software, version 11.0 (SPSS, Chicago, IL, USA).

© 2007 The Japan Society of Hepatology

Table 2 Baseline of hepatitis C virus patients with less than 30 U/L aminotransferase who received antiviral therapy

	PLT ≥ 150 000/μL (group A-1)	PLT < 150 000/μL (group A-2)	*P*-value
No. patients	204	51	
Age	48.4 ± 12.7	58.7 ± 7.5	<0.001*
Sex (male/female)	90/114	22/29	1.000**
BMI (kg/m²)	21.6 ± 3.0	21.3 ± 2.4	0.514*
HOMA-IR	2.8 ± 3.5	1.2 ± 0.8	0.598*
Genotype: 1/2/others	101/101/2	25/26/0	0.952**
Viral load: low/high	112/92	26/25	0.574**
Histology			
F stage (0/1/2/3/4)	29/142/27/6/0	1/25/21/3/1	<0.001**
Grade (0–1/2,3)	179/25	33/18	<0.001**
Fatty change† 0–1/2–4	188/16	44/7	0.582**
Iron load‡ 0/1–4	82/12	17/3	0.762**
Ferritin (ng/mL)	86.0 ± 112.1	73.9 ± 46.6	0.204*
PLT count (/μL)	21.0 ± 4.4	12.1 ± 2.5	<0.001*
Hyaluronate (ng/mL)	41.8 ± 56.1	112.5 ± 109.9	<0.001*
Duration of antiviral therapy (weeks)	25.7 ± 10.3	27.0 ± 9.9	0.503*
Effects of therapy			
SVR/non-SVR	115/89	27/24	0.66**

**P*-values were calculated by Mann–Whitney-*U*-test. **Fisher-exact-test. †0: no fatty change, 1: ≤10%, 2: 11–33%, 3: 34–66%, 4: ≥67% of hepatocyte; ‡no stain by 400×, 1: few stains by 250×, 2: stains by 100×, 3: stains by 25×, 4: stains by 10×. There were significant differences in age ($P < 0.001$), distribution of F stage ($P < 0.001$), grade of inflammatory activity ($P < 0.001$), PLT count ($P < 0.001$), and serum-hyaluronic acid ($P < 0.001$) between groups A-1 and A-2. Frequency of F2–4 patients was 16.2% in group A-1 and 51.6% in group A-2. Values are expressed as mean ± SD.
BMI, body mass index; HOMA-IR, homeostasis model assessment–insulin resistance; PLT, platelet counts; SVR, sustained viral responders.

RESULTS

Demographic, clinical, and histological features of 464 HCV carriers with normal serum ALT

THE CHARACTERISTICS OF the 464 HCV carriers with normal serum ALT are shown in Table 1. There were significant differences in sex, frequency of steatosis, serum ferritin levels, BMI, and the incidence of low PLT counts (<150 000/μL) between groups A and B.

There were significant differences in age, fibrosis (F) stage, inflammatory activity, PLT counts, and serum hyaluronate between groups A-1 and A-2 (Table 2). The frequency of stage F2–4 patients was 16.2% in group A-1, and 49.0% in group A-2 (Table 2). In group B, there were significant differences in age, F stage, PLT counts, and serum hyaluronate between groups B-1 and B-2 (Table 3). There were no F4 patients in group A-1 and B-1, and the frequency of F3 patients was very low compared with those in groups A-2 and B-2 (2.6% *vs* 7.6%). The PLT counts decreased in proportion to the progression of liver fibrosis as follows; F0 ($n = 51$); $20.7 \pm 5.2 \times 10^4/\mu L$, F1 ($n = 288$); $19.8 \pm 5.6 \times 10^4/\mu L$, F2 ($n = 105$); $16.9 \pm 5.3 \times 10^4/\mu L$, F3 ($n = 17$); $15.9 \pm 4.6 \times 10^4/\mu L$, and F4 ($n = 3$); $11.3 \pm 3.8 \times 10^4/\mu L$.

Of the 464 patients, the frequency of the F0–1 stages was 80.1% and that of the F2–4 stages was 19.9% in patients with PLT counts ≥150 000/μL, and it was 50.4% and 49.6%, respectively, in patients with PLT counts <150 000/μL. In patients with PLT counts $\geq 17.0 \times 10^4/\mu L$, 80.8% were in stages F0–1 and 19.2% were in stages F2–4, and in patients with PLT counts $<17.0 \times 10^4/\mu L$, 60.1% were in stages F0–1 and 39.9% were in stages F2–4.

The SVR rates of IFN therapy were 52.4% in F0–1 patients, 49.5% in F2–4 patients ($P = 0.896$ by Fisher's exact test), and 58.0% and 43.8% ($P = 0.592$) in IFN/Riba therapy, respectively.

In patients with genotype 1b and high viral load, the SVR rate was 12.5%. The SVR rate in genotype 2 patients was 60.4% in the IFN group and 67.7% in the IFN/Riba combination therapy group.

© 2007 The Japan Society of Hepatology

Table 3 Baseline of hepatitis C virus carriers with 31–40 U/L aminotransferase who received antiviral therapy

	PLT ≥ 150 000/μL (group B-1)	PLT < 150 000/μL (group B-2)	*P*-value
No. patients	141	68	
Age	48.2 ± 11.9	57.9 ± 7.5	<0.001*
Sex (male/female)	80/61	37/31	0.751**
BMI (kg/m²)	22.9 ± 3.1	22.7 ± 2.6	0.08*
HOMA-IR	3.0 ± 2.0	8.2 ± 9.5	0.8.8*
Genotype: 1/2/others	82/58/1	30/38/0	0.095**
Viral load: low/high	64/77	35/33	0.542**
Histology			
F stage (0/1/2/3/4)	17/91/31/2/0	4/30/26/6/2	<0.001**
Grade (0–1/2,3)	116/25	50/18	0.114**
Fatty change† 0–1/2–4	111/30	50/18	0.10**
Iron load‡ 0/1–4	67/12	30/7	0.762**
Ferritin (ng/mL)	114.4 ± 116.1	127.2 ± 167.8	0.869*
PLT count (/μL)	21.5 ± 4.9	12.2 ± 2.1	<0.001*
Hyaluronate (ng/mL)	46.9 ± 35.4	100.7 ± 0.98.1	<0.001*
Administration of IFN (weeks)	26.1 ± 11.9	27.7 ± 11.4	0.983*
Effects of therapy			
SVR/non-SVR	64/77	35/33	0.409**

**P*-values were calculated by Mann–Whitney-*U*-test. **Fisher-exact-test. †0: no fatty change, 1: ≤10%, 2: 11–33%, 3: 34–66%, 4: ≥67% of hepatocyte; ‡no stain by 400×, 1: few stains by 250×, 2: stains by 100×, 3: stains by 25×, 4: stains by 10×. In group B, there were significant differences in age ($P < 0.001$), distribution of F stage ($P < 0.001$), PLT count ($P < 0.001$), and hyaluronic acid ($P < 0.001$) between B-1 and B-2. Frequency of F2–4 was 23.4% in B-1and 50.0% in B-2, respectively. Values are expressed as mean ± SD.
BMI, body mass index; HOMA-IR, homeostasis model assessment–insulin resistance; IFN, interferon; PLT, platelet counts; SVR, sustained viral responders.

Demographic, clinical, and histological features of 129 HCV carriers with PNALT

The demographic and clinical features of the 129 HCV carriers with PNALT who were followed up for 7.2 years are shown in Table 4. Normal liver histology was noted in 17 patients, 102 showed minimal to mild CH, and 10 had moderate CH. Steatosis was seen in 7% and iron loading was noted in 12%.[18]

Of the 78 patients followed longer than 7 years (mean follow-up period; 10.4 ± 3.1 years), 11 (14%) had continuously normal ALT (G-1), 43 (55%) showed a transient elevation of ALT (G-2), and 24 (31%) changed to CH with continuously elevated ALT (G-3).

Thirty-nine patients received repeated liver biopsies (2–4 times). Of the 39 patients, six were in G-1, 17 were in G-2, and 16 were in G-3. The intervals between the first biopsy and the last biopsy in these three groups were 7.1, 7.8, and 7.2 years, respectively. The progression of the F stage was noted in two of six in G-1, six of 17 in G-2, and seven of 16 in G-3. The median rates of fibrosis progression per year for these three groups were 0.05, 0.05, and 0.08 fibrosis unit. HCC was not detected in any patients during the follow-up periods.

Guidelines for the antiviral therapy of HCV carriers with normal serum ALT focused on the inhibition of the development of HCC

Considering the risk of progression to liver cirrhosis and the development of HCC, as well as the expected efficacy and various side-effects of antiviral therapy, an algorithm is needed for the management of HCV carriers with normal serum ALT. The progression rate of liver fibrosis stage was 0.05/year in HCV carriers with PNALT. The annual incidence of HCC in CH-C patients has been reported to be 0.5% at stages F0–F1, 1–2% at stage F2, 3–5% at stage F3, and 7% at stage F4.[4]

In principle, follow up without antiviral treatment is recommended for HCV carriers with PNALT (ALT ≤30 U/L) and PLT counts ≥150 000/μL, particularly in older patients (i.e. >65 years old), because over 90% show normal or minimal liver damage with good prognoses. However, antiviral therapy is not contraindicated for such patients since roughly 40% are infected with HCV genotype 2,[18] which suggests a high rate of SVR to the therapy with PEG-IFN/Riba.

As for the indication of antiviral therapy for HCV carriers with normal serum ALT (≤40 U/L), the PLT

© 2007 The Japan Society of Hepatology

Table 4 Characteristics of 129 HCV carriers with persistently normal ALT who received liver biopsy

	$n = 129$	Follow up over 5 years ($n = 78$)
Follow-up period (years)	7.2 ± 3.2	10.4 ± 3.1
Age (years)	48 (21–77)	45 (29–71)
Male ($n = 24$)	49.8 ± 16.4	42.3 ± 14.9
Female ($n = 105$)	47.2 ± 12.5	46.6 ± 11.6
Sex (male/female)	24/105	10/68
ALT (U/L)	8–30	9–30
Male ($n = 24$)	22.5 ± 5.7	21.1 ± 5.4
Female ($n = 105$)	21.6 ± 4.8	22.3 ± 5.1
PLT (×10^4/ μL)	15–31	15–31
Ferritin (ng/mL)	5–225	5–225
Male ($n = 24$)	76.2 ± 53.5	84.6 ± 59.2
Female ($n = 105$)	60.0 ± 43.3	66.6 ± 52.5
HCV genotype	G1 ($n = 58$), G2 ($n = 45$)	
	Mixed and unclassified ($n = 16$)	
BMI (kg/m^2)	16–27	16–27
Male	22.2 ± 1.7	21.9 ± 1.9
Female	21.3 ± 2.2	21.0 ± 2.4

Values are expressed as mean ± SD.
ALT, alanine aminotransferase; BMI, body mass index; HCV, hepatitis C virus; PLT, platelet.

count is a good indicator for discriminating as to whether or not they have minimal to mild fibrosis or moderate to advanced fibrosis. Serum hyaluronate levels were significantly higher in HCV carriers with 31–40 U/L ALT having less than 150 000/μL PLT (Table 3). Advanced hepatic F stage, an elevated ALT level, old age (>65 years old), and sex (male) are important risk factors for the development of HCC.[6,18,30] We advocated an algorithm for such patients (Fig. 1) taking into consideration the risk of the progression to cirrhosis and the development of HCC. Therapy with PEG-IFN/Riba is the first-line treatment; therapy for 48 weeks is recommended for genotype 1 patients with high viral load and 12–24 weeks therapy for genotypes 2 and 1 with low viral load.

DISCUSSION

OUR PREVIOUS STUDY in 129 HCV carriers with PNALT demonstrated a predominance of females, higher frequency of genotype 2, minimal to mild liver histology, and very slow progression of hepatic fibrosis.[18] However, over 30% of these patients advanced to CH-C with elevated ALT levels during the 7-year follow up.

There are many reports concerning the natural course of liver fibrosis in CH-C patients, including those who are HCV carriers with normal serum ALT.[19,31–39] More than half of CH-C patients show progression of F stage from F1 to F2–4 within 10 years, and it was reported that the progression of liver fibrosis in HCV carriers with normal serum ALT was more rapid than was observed in the present study.[23] The main reason for the discrepancy between the report by Puoti *et al.*[23] and our results might be due to the definitions used for the normal range of serum ALT. In our previous study, the patients were HCV carriers with PNALT (ALT ≤ 30 U/L) and PLT counts ≥150 000/μL. On the other hand, the patients in the study by Puoti *et al.* had ALT levels ≤40 U/L, irrespective of PLT counts, in which cirrhotic patients might be included.[23] However, recent studies have demonstrated that normal ALT levels are less than 30 U/L[24] or 25 U/L in men[40] and less than 19 U/L[24] or 22 U/L in women.[40]

The present study demonstrated that the different distribution of hepatic F stage became remarkable when the A and B groups were divided into two subgroups according to their PLT counts. In HCV carriers with ALT levels ≤30 U/L, the frequency of stages F2–3 was 16.2% among those with PLT counts ≥150 000/μL; however, the frequency of stages F2–3 was 49.0% in those with PLT counts <150 000/μL. Conversely, in HCV carriers with ALT levels between 31 and 40 U/L, the frequency of stages F2–4 was 23.4% among those with PLT counts ≥150 000/μL and 50.0% in those with PLT counts <150 000/μL. The PLT count is a useful marker in dis-

© 2007 The Japan Society of Hepatology

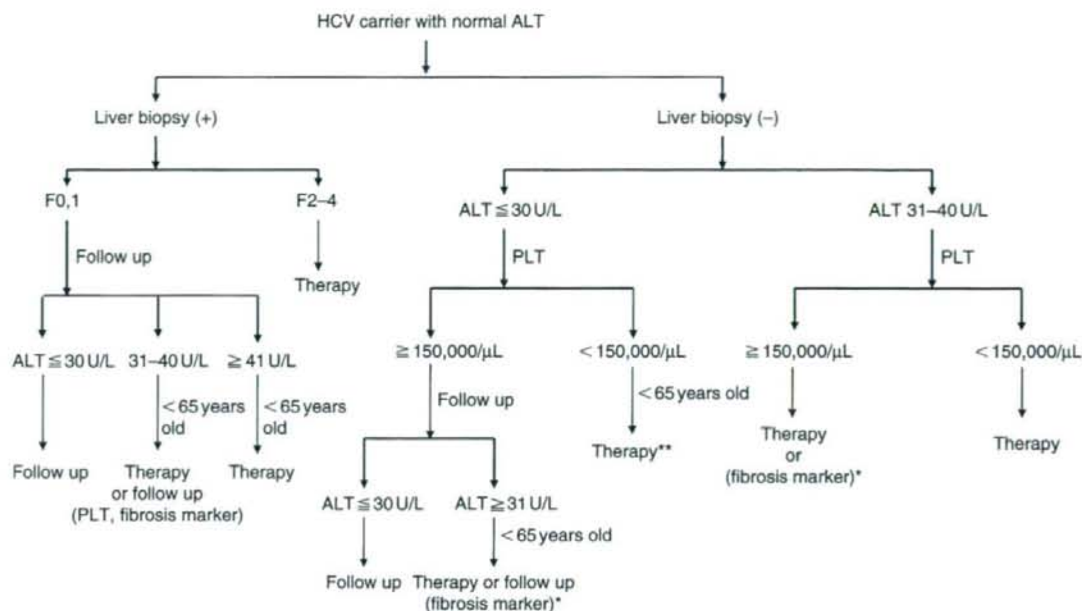

Figure 1 Algorithm for the management of hepatitis C virus (HCV) carriers with normal serum aminotransferase (ALT, ≤40 U/L) focused on the inhibition of the development of hepatocellular carcinoma. In patients who underwent liver biopsy, F0 and F1 patients younger than 65 years are candidates for antiviral therapy, especially those with genotype 2 after the elevation of serum ALT levels. In patients who did not undergo liver biopsy, ALT and platelet (PLT) levels are good indicators for determining candidates for antiviral therapy. Older patients (>65 years) and/or patients having uncontrolled hypertension, diabetes mellitus, or anemia should not be treated with pegylated interferon and ribavirin. Combination therapy with pegylated interferon and ribavirin for 48–72 weeks is recommended for patients with genotype 1 and high viral load, and 12–24 weeks therapy is suggested for patients with genotype 2 and genotype 1 with low viral load. *,**Serum fibrosis markers, such as hyaluronate, might be useful to decide whether patients are candidates for antiviral therapy or not.

criminating between stages F0–1 and F2–4 F in HCV carriers with normal serum ALT (≤40 U/L). In the present study, the mean PLT count in F2 and F3 patients was 16.9 ± 5.3 ($\times 10^4/\mu L$) and 15.9 ± 4.6 ($\times 10^4/\mu L$), respectively. The distribution of the F stage was not significantly different between patients with PLT counts $\geq 15 \times 10^4/\mu L$ versus $< 15 \times 10^4/\mu L$ and $\geq 17 \times 10^4/\mu L$ versus $< 17 \times 10^4/\mu L$.

The SVR rate for genotype 1 patients with high viral load treated with either IFN monotherapy or IFN/Riba were 12.5% and 37.7%, respectively. In genotype 2 patients with high viral load, the SVR rate in the present study was better than the data of Japanese CH-C patients with elevated ALT levels in our previous paper.[6] It was not reasonable to compare the SVR rates between HCV carriers with normal serum ALT and CH-C with elevated ALT in the present study, because the total dosage of IFN and the duration of treatment were significantly different.

The annual incidence of HCC is correlated with the progression of liver fibrosis, that is, the stage of liver disease.[2–4,6] Sustained low serum ALT levels are also associated with a lower incidence of HCC.[2,6,41] PEG-IFN/Riba therapy is expensive and induces various side-effects. The present results indicate that most HCV carriers with normal serum ALT (≤40 U/L) and PLT counts ≥150 000/μL have minimal to mild liver damage, indicating a low risk for the progression to cirrhosis and the development of HCC. This was more remarkable in patients with ALT levels ≤30 U/L and PLT counts ≥150 000/μL. However, nearly half of the patients with PLT count <150 000/μL have F2 or F3 F stages, indicating a certain risk for the progression to cirrhosis and the development of HCC. Fibrosis

© 2007 The Japan Society of Hepatology

progression is associated with age, baseline and follow-up ALT levels, inflammatory activity and steatosis in the initial liver biopsy, and alcohol consumption.[42] The present results indicate that most HCV carriers with PNALT have a good prognosis and a low risk of developing HCC.

Liver biopsy is a useful procedure for identifying the stage of liver fibrosis; however, it is invasive and may sometimes cause complications.[43,44] The error rate of predicting the F stage with this procedure can be estimated to be as high as 20%.[45] Recently introduced biochemical markers, such as FibroTest,[46] and FibroScan,[47–49] are excellent procedures for identifying liver fibrosis stage in CH-C patients.[50] The combined use of FibroScan and FibroTest is useful for accurately estimating moderate to severe liver fibrosis in most patients with CH-C, but not in F0 and F1 patients.[51]

Recently, Alberti proposed an individualized management algorithm for HCV carriers with PNALT with or without liver biopsy in which HCV genotype, patient age, motivation to receive antiviral therapy, and factors influencing side-effects were included.[52] The algorithm using a combination of serum ALT levels and PLT counts in the present study is simple, but it is useful because it focuses mainly on the inhibition of the progression to cirrhosis and the development of HCC.

ACKNOWLEDGMENTS

THIS PROJECT WAS supported in part by a grant-in-aid from the Ministry of Health, Labour and Welfare of Japan. Twelve hepatologists were from the Japanese Study Group of the Standard Antiviral Therapy for Viral Hepatitis (chief: Hiromitsu Kumada, Toranomon Hospital).

REFERENCES

1 Kasahara A, Hayashi N, Mochizuki K *et al.* Risk factors for hepatocellular carcinoma and its incidence after interferon treatment in patients with chronic hepatitis C. Osaka Liver Disease Study Group. *Hepatology* 1998; **27**: 1394–402.

2 Okanoue T, Itoh Y, Minami M *et al.* Interferon therapy lowers the rate of progression to hepatocellular carcinoma in chronic hepatitis C but not significantly in advanced stage: a retrospective study in 1148 patients. *J Hepatol* 1999; **30**: 653–9.

3 Ikeda K, Saitoh S, Arase Y *et al.* Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis C: a long-term observation study of 1643 patients using statistical bias correction with proportional hazard analysis. *Hepatology* 1999; **29**: 11–19.

4 Yoshida H, Shiratori Y, Moriyama M *et al.* Interferon therapy reduced the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of hepatocarcinogenesis by interferon therapy. *Ann Inter Med* 1999; **131**: 174–81.

5 Tanaka H, Tsukuma H, Kasahara A *et al.* Effect of interferon therapy on the incidence of hepatocellular carcinoma and mortality of patients with chronic hepatitis C: retrospective cohort study of 738 patients. *Int J Cancer* 2000; **87**: 741–9.

6 Okanoue T, Itoh Y, Kirishima T *et al.* Transient biochemical response in interferon therapy decreases the development of hepatocellular carcinoma for five years and improves the long-term survival of chronic hepatitis C patients. *Hepatol Res* 2002; **23**: 62–77.

7 Serfaty L, Nousbaum JB, Elghouzzi MH, Giral P, Legendre C, Poupon R. Prevalence, severity, and risk factors of liver disease in blood donors positive in a second-generation anti-hepatitis C virus screening test. *Hepatology* 1995; **21**: 330–7.

8 Piton A, Poynard T, Imbert-Bismut F *et al.* Factors associated with serum alanine transaminase activity in healthy subjects: consequences for patients with chronic hepatitis C. *Hepatology* 1998; **27**: 1213–19.

9 Alberti A, Noventa F, Benvegnu L, Boccata S, Gatta A. Prevalence of liver disease in a population of asymptomatic individuals with hepatitis C virus infection. *Ann Intern Med* 2002; **137**: 961–4.

10 Tassopoulos NC. Treatment in patients with normal ALT levels. European Association for the Study of the Liver (EASL) International Conference on Hepatitis C, Paris, February 26–27, 1999. *J Hepatol* 1999; **30**: 956–61.

11 Marcellin P, Levy S, Erlinger S. Therapy of hepatitis C: patients with normal aminotransferase levels. *Hepatology* 1997; **26**: 133S–6.

12 Jamal MM, Soni A, Quinn PG, Wheeler DE, Arora S, Johnston DE. Clinical features of hepatitis C-infected patients with persistently normal alanine transaminase levels in the Southwestern United States. *Hepatology* 1999; **30**: 1307–11.

13 Nutt AK, Hassan HA, Lindsey J, Lamps LW, Raufman JP. Liver biopsy in the evaluation of patients with chronic hepatitis C who have repeatedly normal or near-normal serum alanine aminotransferase levels. *Ann Intern Med* 2000; **109**: 62–4.

14 Pradat P, Alberti A, Poynard T *et al.* Predictive value of ALT levels for histologic findings in chronic hepatitis C: a European collaborative study. *Hepatology* 2002; **36**: 973–7.

15 Hui CK, Monto A, Belaye T, Lau E, Wright TL. Outcomes of interferon alpha and ribavirin treatment for chronic hepatitis C in patients with normal serum aminotransferase. *Gut* 2003; **52**: 1644–8.

16 Renou C, Halfon P, Pol S *et al.* Histological features and HLA class II alleles in hepatitis C virus chronically infected

© 2007 The Japan Society of Hepatology

patients with persistently normal alanine aminotransferse levels. *Gut* 2002; **51**: 585–90.

17 Okanoue T, Yasui K, Sakamoto S *et al.* Circulating HCV RNA, HCV genotype, and liver histology in asymptomatic individuals reactive for anti-HCV antibody and their follow-up study. *Liver* 1996; **16**: 241–7.

18 Okanoue T, Makiyama A, Nakayama M *et al.* A follow-up study to determine the value of liver biopsy and need for antiviral therapy for hepatitis C virus carriers with persistently normal serum aminotransferase. *J Hepatol* 2005; **43**: 599–605.

19 Bacon BR. Treatment of patients with hepatitis C and normal serum aminotransferase levels. *Hepatology* 2002; **36**: S179–84.

20 Zeuzem S, Diago M, Gane E *et al.* Peginterferon alfa-2a (40 kilodaltons) and ribavirin in patients with chronic hepatitis C and normal aminotransferase levels. *Gastroenterology* 2004; **127**: 1724–32.

21 Manns MP, McHutchison JG, Gordon SC *et al.* Peginterferon alfa-2b plus ribavirin compared to interferon alfa-2b plus ribavirin for the treatment of chronic hepatitis C: a randomized controlled trial. *Lancet* 2001; **359**: 958–65.

22 Fried MW, Shiffman ML, Reddy KR *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975–82.

23 Puoti C, Magrini A, Stati T *et al.* Clinical, histological, and virological features of hepatitis C virus carriers with persistently normal or abnormal alanine aminotransferase levels. *Hepatology* 1997; **26**: 1393–8.

24 Prati D, Taidoli E, Zanela A *et al.* Updated definition of healthy ranges for serum alanine aminotransferase levels. *Ann Intern Med* 2002; **137**: 1–9.

25 Desmet VJ, Gerber M, Hoofnagle JH *et al.* Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994; **19**: 1513–20.

26 Ishak K, Baptista L, Bianchi L *et al.* Histological grading and staging of chronic hepatitis. *J Hepatol* 1995; **22**: 696–9.

27 MacSween RNM, Anthony PP, Sheuer PJ. *Pathology of the Liver*. Edinburgh: Churchill Livingstone, 1987.

28 Tsukiyama-Kohara K, Yamaguchi K, Maki N *et al.* Antigenicities of group 1 and 2 hepatitis C virus polypeptides: molecular basis of diagnosis. *Virology* 1993; **192**: 430–7.

29 Simmonds P, Alberti A, Alter HJ *et al.* A proposed system for the nomenclature of hepatitis C virus genotypes. *Hepatology* 1994; **19**: 1321–4.

30 Makiyama A, Itoh Y, Kasahara A *et al.* Characteristics of patients with chronic hepatitis C who develop hepatocellular carcinoma after sustained response to interferon. *Cancer* 2004; **101**: 1616–22.

31 Healey CJ, Chapman RWG, Fleming KA. Liver histology in hepatitis C virus infection: a comparison between patients with persistently normal or abnormal transaminase. *Gut* 1993; **37**: 274–8.

32 Ohkoshi S, Tawaraya H, Kuwana K *et al.* A retrospective study of hepatitis C virus carriers in a local endemic town in Japan. *Dig Dis Sci* 1995; **40**: 465–71.

33 Puoti C, Castellacci R, Montagness F *et al.* Histological and virological features and follow-up of HCV carriers with normal aminotransferase levels: the Italian Study of the Asymptomatic C Carriers (ISACC). *J Hepatol* 2002; **37**: 117–23.

34 Yano M, Kumada H, Kage M *et al.* The long-term pathological evolution of chronic hepatitis C. *Hepatology* 1996; **332**: 1463–6.

35 Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINVIR, and DOSVIRC. *Lancet* 1997; **346**: 825–32.

36 Takahashi M, Yamada G, Miyamoto R, Doi H, Endo H, Tsuji T. Natural course of chronic hepatitis C. *Am J Gastroenterol* 1993; **88**: 240–3.

37 Ghany MG, Kleiner DE, Alter H *et al.* Progression of fibrosis in chronic hepatitis C. *Gastroenterology* 2003; **124**: 97–104.

38 Mathurin P, Moussalli J, Cardanei J-F *et al.* Slow progression rate of fibrosis in hepatitis C virus patients with persistently normal alanine transaminase activity. *Hepatology* 1998; **27**: 868–72.

39 Hui C-K, Belaye T, Montegrade K, Wright TL. A comparison in the progression of liver fibrosis in chronic hepatitis C between persistently normal and elevated transaminase. *J Hepatol* 2003; **38**: 511–17.

40 Prati D, Shiffman ML, Diago M *et al.* Viral and metabolic factors influencing alanine aminotransferase activity in patients with chronic hepatitis C. *J Hepatol* 2006; **44**: 679–85.

41 Tarao K, Ohkawa S, Tamai S, Miyakawa K. Sustained low serum GPT level below 80 INU in HCV- associated cirrhotic patients by multiagents prevent development of hepatocellular carcinoma. *Cancer* 1994; **73**: 1149–54.

42 Boccato S, Pistis R, Noventa F, Guido M, Benvegnu L, Alberti A. Fibrosis progression in initially mild chronic hepatitis C. *J Viral Hepat* 2006; **13**: 297–302.

43 Cadranel JF, Rufat P, Degos F. Practices of liver biopsy in France: results of a prospective nationwide survey. For the Group of Epidemiology of the French Association for the Study of the Liver (AFEF). *Hepatology* 2000; **32**: 477–81.

44 Castera L, Negre I, Samii K, Buffet C. Pain experienced during percutaneous liver biopsy. *Hepatology* 1999; **30**: 1529–30.

45 Afdal NH. Diagnosing fibrosis in hepatitis C: is the pendulum swinging from biopsy to blood test? *Hepatology* 2003; **37**: 972–4.

46 Imbert-Bismut F, Ratziu V, Pieroni L, Charlotte F, Benhamou Y, Poynard T. Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. *Lancet* 2001; **357**: 1069–75.

© 2007 The Japan Society of Hepatology

47 Sandrin L, Fourquet B, Hasquenoph JM *et al.* Transient elastography: a new invasive method for assessment of hepatic fibrosis. *Ultrasound Med Biol* 2003; **29**: 1705–13.

48 Castera L, Vergniol J, Foucher J *et al.* Prospective comparison of transient elastography, fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. *Gastroenterology* 2005; **128**: 343–50.

49 Saito H, Tada S, Nakamoto N *et al.* Efficacy of non-invasive elastometry on staging of hepatic fibrosis. *Hepatol Res* 2004; **29**: 97–103.

50 Colletta C, Smirne C, Fabris C *et al.* Value of two noninvasive methods to detect progression of fibrosis among HCV carriers with normal aminotransferase. *Hepatology* 2005; **42**: 838–645.

51 Castera L, Foucher J, Bertet J, Couzigou P. FibroScan and FibroTest to assess liver fibrosis in HCV with normal aminotransferse. *Hepatology* 2006; **43**: 373–4.

52 Alberti A. Towards more individualized management of hepatitis V virus patients with initially or persistently normal alanineaminotransferase levels. *J Hepatol* 2005; **42**: 266–74.

© 2007 The Japan Society of Hepatology

2008年7月作成
B0023
EM0708BP

doi: 10.1111/j.1872-034X.2007.00280.x

Original Article

Evidence of oxidative stress as a cofactor in the development of insulin resistance in patients with chronic hepatitis C

Hironori Mitsuyoshi,[1] Yoshito Itoh,[1] Yoshio Sumida,[2] Masahito Minami,[1] Kouichirou Yasui,[1] Toshiaki Nakashima[3] and Takeshi Okanoue[4]

[1]*Molecular Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto,* [2]*Department of Gastroenterology and Hepatology, Nara City Hospital, Nara,* [3]*Saiseikai Kyoto Hospital, Kyoto, and* [4]*Saiseikai Suita Hospital, Suita, Japan*

Aim: The mechanisms by which metabolic disorders develop in patients with chronic hepatitis C are unknown. Our study aimed to test whether oxidative stress contributes to these mechanisms.

Methods: The index of homeostasis model assessment–insulin resistance (HOMA–IR) and serum and hepatic levels of thioredoxin (Trx), which are markers of oxidative stress, were evaluated in 203 biopsy-proven chronic hepatitis C patients with hepatitis C virus (HCV) genotype 1 or 2 infection. HOMA–IR and Trx levels were compared with baseline values after phlebotomy in 23 patients.

Results: HOMA–IR and serum Trx levels were significantly correlated with disease stage (HOMA–IR, $P < 0.00001$; Trx, $P < 0.0001$) and independently predicted fibrosis scores (HOMA–IR, $P < 0.05$; Trx, $P < 0.005$). Steatosis (%) was significantly correlated with HOMA–IR ($P < 0.00005$) and Trx ($P < 0.001$) stage ($P < 0.00001$). Serum Trx levels were significantly correlated with HOMA–IR ($P < 0.05$), even after adjustment for body mass index ($P < 0.05$). Furthermore, the mRNA levels of hepatic Trx were significantly correlated with HOMA–IR ($P < 0.05$) and independently-predicted HOMA–IR ($P < 0.05$). The alanine aminotransferase ($P < 0.00001$), Trx ($P < 0.05$), and HOMA–IR ($P < 0.05$) serum levels decreased significantly after phlebotomy; these effects were similar even in non-responders to interferon.

Conclusion: Oxidative stress contributed to the development of IR irrespective of obesity in patients with HCV genotype 1 or 2 infection. This study could contribute to our understanding of how metabolic disorders develop and how they should be treated in chronic hepatitis C patients.

Key words: hepatitis C virus, insulin resistance, oxidative stress, steatosis, thioredoxin

INTRODUCTION

CHRONIC HEPATITIS C progresses to cirrhosis and eventually to hepatocellular carcinoma (HCC).[1] Although interferon (IFN)-based antiviral therapy has achieved great advances, it can not eradicate hepatitis C virus (HCV) in approximately 50% of patients infected with the genotype 1 strain,[1] which is highly prevalent in Japan. Therefore, other therapeutic strategies remain important, and efforts to understand the pathogenesis are required.

Metabolic disorders have recently been implicated in the pathogenesis of chronic hepatitis C.[2–6] HCV-infected patients with hepatic steatosis exhibit clinical features associated with metabolic syndromes,[3] and glucose intolerance is considered to represent an extra-hepatic manifestation of HCV infection.[2,4] Furthermore, grades of steatosis are reported to predict rapid fibrosis progression,[5] and diabetes increases the risk of HCC.[6] From these findings, insulin resistance (IR), a central cause of metabolic syndromes,[7] has been described as a risk factor in advanced staged chronic hepatitis C patients,[2,8] as seen in non-alcoholic steatohepatitis.[9] Thus, insulin signaling could be an important target for

Correspondence: Assistant Professor Hironori Mitsuyoshi, Molecular Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-8566, Japan. Email: hmitsu@koto.kpu-m.ac.jp
Received 20 June 2007; revision 3 September 2007; accepted 5 September 2007.

© 2007 The Japan Society of Hepatology

the management of patients with HCV infection; however, how IR develops is not well understood.

In this study, we focused on the role of oxidative stress, another key player in progressive liver injury in patients with chronic hepatitis C infection[10] in the development of IR. Because steatosis results in the overproduction of reactive oxygen species (ROS),[11] and ROS may exacerbate hepatic insulin sensitivity,[12] we hypothesized a close relationship between IR and oxidative stress. Therefore, we retrospectively analyzed the index of IR[13] and the serum and hepatic levels of thioredoxin (Trx), which are markers of oxidative stress,[14] in 203 patients with HCV infection. We also investigated whether relieving hepatic oxidative stress could improve IR among these patients.

METHODS

Patients

CHRONIC HEPATITIS PATIENTS who underwent liver biopsies in our institute between April 2003 and March 2006 were selected according to the following criteria: no excessive alcohol intake (more than 40 g/week), as assessed by interview (at least on 3 occasions); positive serum HCV-RNA, as confirmed by reverse transcription–polymerase chain reaction (RT–PCR); infection with HCV genotype 1 (1a, 1b) or 2 (2a, 2b); no history of antiviral therapy nor treatment with steatosis-inducing drugs within the 12 months before the study; negativity for hepatitis B surface antigen or antibodies to HIV; and an absence of other forms of chronic liver disease. Anthropometry and laboratory data were collected from all patients at the time of the liver biopsy. The serum HCV-RNA level was determined using the AMPLICOR GT HCV Monitor (Roche Diagnostic Systems, Tokyo, Japan). HCV genotypes 1 and 2 were determined by a serologic genotyping assay.[15] Serogroups 1 and 2 in this assay correspond to genotypes 1 (1a, 1b) and 2 (2a, 2b). Informed written consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki approved by the Ethics Committee of the Kyoto Prefectural University of Medicine.

Laboratory determination

After a 12-h overnight fast, venous blood samples were drawn to determine alanine aminotransferase (ALT), γ-glutamylcysteine transpeptidases, fasting plasma glucose (FPG), insulin (IRI), triglyceride, and ferritin levels. These parameters were measured using standard techniques from clinical chemistry laboratories. The index of IR was calculated only in patients without overt diabetes (FPG >126 mg/dL), according to the homeostasis model assessment (HOMA).[13] The formula for IR was as follows: HOMA–IR = FPG (mg/dL) × IRI (μU/mL)/405. HOMA–IR was only calculated in patients without diabetes ($n = 189$).

Measurement of Trx

The levels of serum Trx were used as a marker of hepatic oxidative stress, as reported previously.[14] For the measurements of Trx concentrations, serum and liver biopsy specimens were stored at −80°C until use. The serum Trx levels were then measured using a commercial, sensitive enzyme-linked immunosorbent assay kit (REDOX BIOSCIENCE, Kyoto, Japan), as described previously.[14] All measurements were made in duplicate and average values were used for the statistical analysis.

The hepatic levels of Trx were measured by real-time PCR. Total RNA was isolated from biopsy specimens using the RNeasy kit (Qiagen, Hilden, Germany). The PCR mixture contained first-strand cDNA and specific primers for human Trx: sense, 5′-CTGCTTTTCAG GAAGCCTTG-3′ and antisense, 5′-ACCCACCTTTTGT CCCTTCT-3′. PCR was performed using the Light Cycler 2.0 System (Roche, Mannheim, Germany), and the mRNA levels of Trx were normalized to those of β-actin.

Histological evaluation

Formalin-fixed and paraffin-embedded liver biopsy specimens were stained with hematoxylin–eosin, Masson's trichrome, and Perl's Prussian blue. Degrees of hepatic fibrosis (stage) were scored as follows: F0 = none, F1 = portal expansion, F2 = bridging fibrosis, F3 = bridging fibrosis with lobular distortion, and F4 = cirrhosis. Degrees of inflammation (grade) were scored as follows: A0 = none, A1 = mild, A2 = moderate, and A3 = severe. Steatosis was assessed according to the percentage of hepatocytes containing fat droplets. The degree of iron loading was graded using a Perl's score of 0–4, as described previously.[16]

Phlebotomy

Phlebotomy was initiated to relieve iron-induced oxidative stress in 23 patients. All patients showed elevated serum ferritin levels and/or persistent abnormal ALT levels, and none showed anemia (hemoglobin <11.0 g/dL). They underwent phlebotomy (300–400 mL) either biweekly or monthly until serum ferritin levels were <20 ng/mL. Thereafter, the serum Trx levels and HOMA–IR were compared with baseline values in each

© 2007 The Japan Society of Hepatology