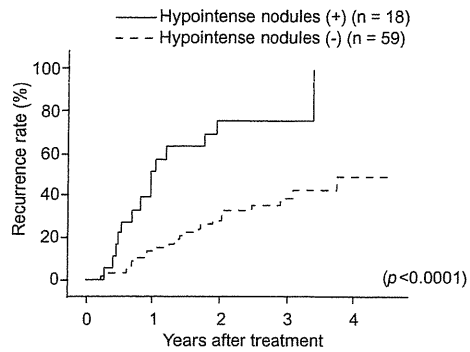

Patients at risk					
Hypointense nodules (+)	18	17	14	7	2
Hypointense nodules (-)	59	58	47	29	16

Fig. 1. Overall recurrence rate after hepatectomy in patients with or without concurrent non-hypervascular hypointense hepatic nodules detected during the hepatobiliary phase of preoperative Gd-EOB-DTPA-enhanced MRI.

MR fluoroscopic bolus detection of the descending aorta (Bolus Trak; Philips Medical Systems). The mean delay times (time interval between the start of bolus administration and the start of image acquisition) for the arterial, portal, and delayed phases were 20, 60, and 180 s, respectively. Immediately after the dynamic study, a respiration-triggered single-shot T2-weighted sequence, with a reduction factor of 4 (1200/100; flip angle, 90°; matrix size, 400 × 512) with 7-mm section thickness, a 1-mm intersection gap, and a 38-cm field of view, was obtained with SPIR. The 20-min-delayed hepatobiliary phase [19] was obtained with a T1-weighted TFE sequence (TR/TE, 4/1.8; flip angle, 12°; matrix size, 256 × 512) with 3.5-mm section thickness, a 0-mm intersection gap, and a 38-cm field of view. All the sequences were obtained with parallel imaging (SENSE). Hypointense hepatic nodules during the hepatobiliary phase of Gd-EOB-DTPA-enhanced MRI were nodules greater than 3.5 mm with low-intensity.

Prior to hepatectomy, all patients underwent CT during hepatic arteriography (CTHA) [20–22] to evaluate the intranodular blood supply, and to confirm the hypervascularity of HCC lesions and the lack of hypervascularity of non-hypervascular hepatic nodules.

All imaging findings were evaluated by a radiologist (Y.S.) and a hepatologist (H.T.) independently, blind to the clinical data. When the imaging assessment was discordant between two reviewers, consensus was made through the discussion.

Statistical analyses

Differences in percentages between groups were analyzed using the Chi-square test. Differences in mean quantitative values were analyzed by the Mann–Whitney *U* test. The date of hepatectomy was defined as time zero for calculations of recurrence rates. In the analysis of the overall recurrence rate, patients in whom HCC did not recur were censored, and those in whom HCC recurred were not censored. In the analysis of the intrahepatic metastasis recurrence rate, patients in whom HCC did not recur or patients with multicentric HCC recurrence were censored, and those in whom HCC recurred as intrahepatic metastases were not censored. In the analysis of the multicentric recurrence rate, patients in whom HCC did not recur were censored and patients with multicentric HCC recurrence were not censored, while those in whom HCC recurred as intrahepatic metastases were excluded from the analysis. The Kaplan–Meier method [23] was used to calculate recurrence rates, and the log-rank test [24] was used to analyze differences.

The Cox proportional hazards model [25] was used for univariate and multivariate analyses of factors related to recurrence. Variables analyzed included patient age and sex, Child-Pugh class (A/B), tumor size, number of tumors (single/multiple), differentiation of resected HCC (well-differentiated/moderately or

Table 2. Univariate and multivariate analyses of factors associated with post-operative recurrence in HCC patients (n = 77).

Factor	Univariate analysis		Multivariate analysis	
	Risk ratio (95% CI)	*p* value	Risk ratio (95% CI)	*p* value
Age	0.9943 (0.9535-1.0396)	0.7974	-	
Sex				
Male	1			
Female	1.0068 (0.6818-1.4290)	0.9711	-	
Child-Pugh class*				
A	1			
B	0.0428 (0.0198-1.5669)	0.2068	-	
Tumor size	0.9376 (0.7179-1.1700)	0.5935	-	
Number of tumors				
Single	1			
Multiple	1.0419 (0.5669-1.6643)	0.8792	-	
Differentiation**				
Well-	1		1	
Moderately/poorly	1.5871 (1.0958-2.4354)	0.0134	1.6536 (1.1381-2.5445)	0.0073
Growth pattern**				
Expansive	1			
Infiltrative	1.1101 (0.6798-1.6625)	0.6487	-	
Portal vein invasion**				
Absent	1		1	
Present	1.5659 (1.0161-2.2813)	0.0428	1.7818 (1.1388-2.6597)	0.0134
Non-hypervascular hypointense nodules				
Absent	1		1	
Present	1.9396 (1.3615-2.7222)	0.0004	2.1767 (1.5089-3.1105)	0.0001

CI, confidence interval.
* Child-Pugh class A includes patients without cirrhosis.
** Evaluated by pathologic examination of resected specimens.

Please cite this article in press as: Toyoda H et al. Non-hypervascular hypointense nodules detected by Gd-EOB-DTPA-enhanced MRI are a risk factor for recurrence of HCC after hepatectomy. J Hepatol (2013), http://dx.doi.org/10.1016/j.jhep.2013.01.030

Research Article

poorly differentiated), growth pattern of resected HCC (expansive growth/infiltrative growth), portal vein invasion of resected HCC (absent/present), and presence of non-hypervascular hypointense nodules on the hepatobiliary phase of Gd-EOB-DTPA-enhanced MRI (absent/present). Data analyses were performed using JMP statistical software, version 6.0 (Macintosh version; SAS Institute, Cary, NC). All p values were derived from 2-tailed tests, with $p < 0.05$ accepted as statistically significant.

Results

Patients characteristics and imaging findings

Patients consisted of 56 males and 21 females with a mean age of 68.3 ± 7.6 years (range, 46–82 years). A total of 40 non-hypervascular hypointense hepatic nodules were identified during the hepatobiliary phase of Gd-EOB-DTPA-enhanced MRI in 28 of 77 patients (36.4%). The size of non-hypervascular hypointense nodules was 1.17 ± 0.38 cm (range, 0.4–2.1 cm). Two of 40 non-hypervascular hypointense hepatic nodules (5.0%) were identified by T2-weighted sequence as high-intensity nodules. The other 38 non-hypervascular hypointense hepatic nodules were not identified either by T1- and T2-weighted sequences. Two nodules were located in segment II of the liver, 7 in III, 1 in IV, 10 in V, 6 in VI, 4 in VII, and 10 in VIII, respectively. Among 28 patients with non-hypervascular hypointense nodules, 19 patients had one non-hypervascular hypointense nodule, 6 patients had 2 nodules, and the remaining 3 patients had 3 nodules. Non-hypervascular hypointense nodules were resected along with HCC lesions during hepatectomy in 10 patients, because they were included within the intended area of resection. Therefore, we categorized these 10 patients and the 49 patients in whom non-hypervascular hypointense nodules were not detected by preoperative Gd-EOB-DTPA-enhanced MRI as the hypointense nodule (−) group and the remaining 18 patients who had residual hypointense nodules after hepatectomy as the hypointense nodule (+) group. Of 13 hypointense nodules in 10 patients resected along with HCC at hepatectomy, 3 nodules were diagnosed as well-differentiated HCC and the remaining 10 nodules were diagnosed as dysplastic nodules on pathologic examination.

Table 1 compares the preoperative characteristics of the study patients. No differences were found in patient age and sex, etiology, liver function, and tumor progression as evaluated by preoperative imaging examinations and by post-operative pathologic examinations. Multiple HCC nodules were resected in 6 patients (10.2%) of the hypointense nodule (−) group and 3 patients (16.7%) of the hypointense nodule (+) group, without difference in proportions. No difference was observed in the length of follow-up period.

Recurrence rate after hepatectomy according to the presence of non-hypervascular hypointense nodules detected during preoperative gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid-enhanced MRI

We determined the recurrence rate in patients after hepatectomy with curative intent based on the presence of non-hypervascular hypointense hepatic nodules identified during the hepatobiliary phase of Gd-EOB-DTPA-enhanced MRI (Fig. 1). The recurrence rate was significantly higher in patients in the hypointense nodule (+) group than in the hypointense nodule (−) group ($p < 0.0001$). In univariate analysis, HCC differentiation and portal vein invasion were identified as factors associated with the rate of recurrence after hepatectomy along with preoperative non-hypervascular hypointense nodules by Gd-EOB-DTPA-enhanced MRI. In multivariate analysis, these factors were confirmed to be independently associated with the rate of recurrence (Table 2). Among 18 patients in the hypointense nodule (+) group, recurrence was observed in 7 of 11 patients with one non-hypervascular hypointense nodule, whereas recurrence was observed in all 7 patients with multiple non-hypovascular hypointense nodules.

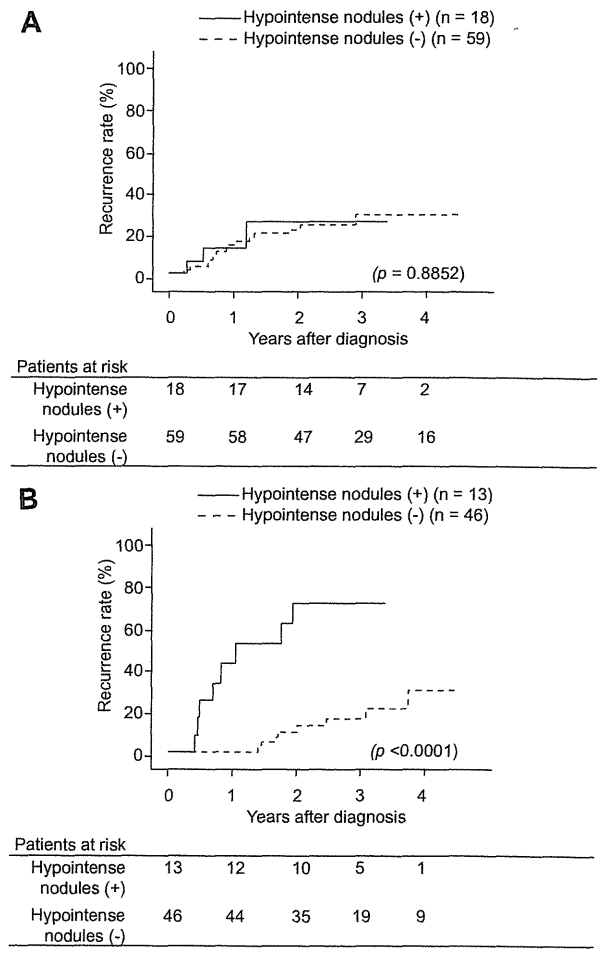

Fig. 2. Recurrence rate after hepatectomy according to the patterns of recurrence. (A) Rates of intrahepatic metastasis recurrence after hepatectomy in patients with or without concurrent non-hypervascular hypointense hepatic nodules detected during the hepatobiliary phase of preoperative Gd-EOB-DTPA-enhanced MRI. (B) Rates of multicentric recurrence after hepatectomy in patients with or without concurrent non-hypervascular hypointense hepatic nodules detected during the hepatobiliary phase of preoperative Gd-EOB-DTPA-enhanced MRI, among 59 patients, excluding 16 patients with intrahepatic metastasis recurrence.

Please cite this article in press as: Toyoda H et al. Non-hypervascular hypointense nodules detected by Gd-EOB-DTPA-enhanced MRI are a risk factor for recurrence of HCC after hepatectomy. J Hepatol (2013), http://dx.doi.org/10.1016/j.jhep.2013.01.030

Table 3. Univariate and multivariate analyses of factors associated with post-operative intrahepatic metastasis recurrence in HCC patients (n = 77).

Factor	Univariate analysis		Multivariate analysis	
	Risk ratio (95% CI)	*p* value	Risk ratio (95% CI)	*p* value
Age	0.9825 (0.9265-1.0470)	0.5743	-	
Sex				
Male	1			
Female	0.9022 (0.4784-1.5192)	0.7148	-	
Child-Pugh class*				
A	1			
B	0.0242 (0.0059-2.1819)	0.3573	-	
Tumor size	1.0051 (0.6929-1.3406)	0.9755	-	
Number of tumors				
Single	1			
Multiple	0.7038 (0.1655-1.5643)	0.4504	-	
Differentiation**				
Well-	1		1	
Moderately/poorly	1.7843 (1.0185-3.7176)	0.0424	1.6742 (0.9520-3.4993)	0.0769
Growth pattern**				
Expansive	1			
Infiltrative	0.9266 (0.3678-1.7453)	0.8365	-	
Portal vein invasion**				
Absent	1		1	
Present	2.1224 (1.2405-3.4608)	0.0079	2.0041 (1.1672-3.2828)	0.0138
Non-hypervascular hypointense nodules				
Absent	1			
Present	1.0474 (0.5012-1.8442)	0.8864	-	

CI, confidence interval.
* Child-Pugh class A includes patients without cirrhosis.
** Evaluated by pathologic examination of resected specimens.

Patterns of recurrence after hepatectomy according to the presence of non-hypervascular hypointense nodules detected during preoperative gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid-enhanced MRI

Among 30 patients with HCC recurrence after hepatectomy, 16 patients (53.3%) had intrahepatic metastasis recurrence and 14 patients (46.7%) had multicentric recurrence. There was no difference in the rate of intrahepatic metastasis recurrence between patients in the hypointense nodule (+) group and those in the hypointense nodule (−) group ($p = 0.8852$). In contrast, patients in the hypointense nodule (+) group had a significantly higher rate of multicentric recurrence than patients in the hypointense nodule (−) group ($p < 0.0001$, Fig. 2). Univariate and multivariate analyses revealed that portal vein invasion was independently associated with intrahepatic metastasis recurrence but preoperative non-hypervascular hypointense nodules detected by Gd-EOB-DTPA-enhanced MRI was not associated with intrahepatic metastasis recurrence (Table 3). The presence of preoperative non-hypervascular hypointense nodules detected by Gd-EOB-DTPA-enhanced MRI was the only factor associated with multicentric recurrence in univariate and multivariate analyses (Table 4). Among 8 HCCs that recurred multicentrically in the hypointense nodule (+) group, 6 nodules (75.0%) had existed as non-hypervascular hypointense hepatic nodules on Gd-EOB-DTPA-enhanced MRI before hepatectomy and progressed to hypervascular HCC tumors (Fig. 3), while the other 2 nodules (25.0%) newly occurred as multicentric recurrence after hepatectomy.

Discussion

Although one study reported that dysplastic nodules and early, well-differentiated HCC can be differentiated based on findings on Gd-EOB-DTPA uptake [26], differentiation of early, non-hypervascular HCC from dysplastic nodules within hypointense nodules is not actually feasible and controversial [27]. In addition, it is nearly impossible to characterize these hepatic nodules specifically using US or MDCT. Therefore, a histological diagnosis should be obtained with percutaneous liver biopsy under US guidance. However, this is not always possible due to the need for multiple samples and its invasive nature. Therefore, we did not resect these hepatic nodules during hepatectomy, except for nodules located within the hepatectomy field.

This study demonstrates a higher rate of recurrence of HCC in patients in whom non-hypervascular hypointense hepatic nodules were identified during the hepatobiliary phase of preoperative Gd-EOB-DTPA-enhanced MRI. This large difference in the recurrence rates indicated that the presence of non-hypervascular hypointense nodules detected during the hepatobiliary phase of Gd-EOB-DTPA-enhanced MRI is a risk factor for recurrence of HCC after hepatectomy. Although we did not find differences in the rate of intrahepatic metastasis recurrence according to the non-hypervascular hypointense hepatic nodule status, we found a significantly higher rate of multicentric recurrence in patients with preoperative concurrent non-hypervascular hypointense hepatic nodules. In addition, the majority of multicentric recurrences involved the hypervascularization of non-hypervascular

Please cite this article in press as: Toyoda H et al. Non-hypervascular hypointense nodules detected by Gd-EOB-DTPA-enhanced MRI are a risk factor for recurrence of HCC after hepatectomy. J Hepatol (2013), http://dx.doi.org/10.1016/j.jhep.2013.01.030

Research Article

Table 4. Univariate and multivariate analyses of factors associated with post-operative multicentric recurrence in HCC patients (n = 59).

Factor	Univariate analysis		Multivariate analysis	
	Risk ratio (95% CI)	*p* value	Risk ratio (95% CI)	*p* value
Age	1.0047 (0.9359-1.0823)	0.8985	-	
Sex				
Male	1			
Female	1.0701 (0.5999-1.7781)	0.8038	-	
Child-Pugh class*				
A	1			
B	0.0664 (0.0176-5.7947)	0.7029	-	
Tumor size	0.9517 (0.6300-1.2943)	0.7801	-	
Number of tumors				
Single	1			
Multiple	1.1331 (0.4469-2.1714)	0.7510	-	
Differentiation**				
Well-	1			
Moderately/poorly	1.5198 (0.8959-2.8769)	0.1249	-	
Growth pattern**				
Expansive	1			
Infiltrative	1.3486 (0.7124-2.2884)	0.3270	-	
Portal vein invasion**				
Absent	1			
Present	1.2908 (0.5077-2.4730)	0.5312	-	
Non-hypervascular hypointense nodules				
Absent	1		1	
Present	2.8436 (1.6900-4.8407)	0.0002	2.8436 (1.6900-4.8407)	0.0002

CI, confidence interval.
* Child-Pugh class A includes patients without cirrhosis.
** Evaluated by pathologic examination of resected specimens.

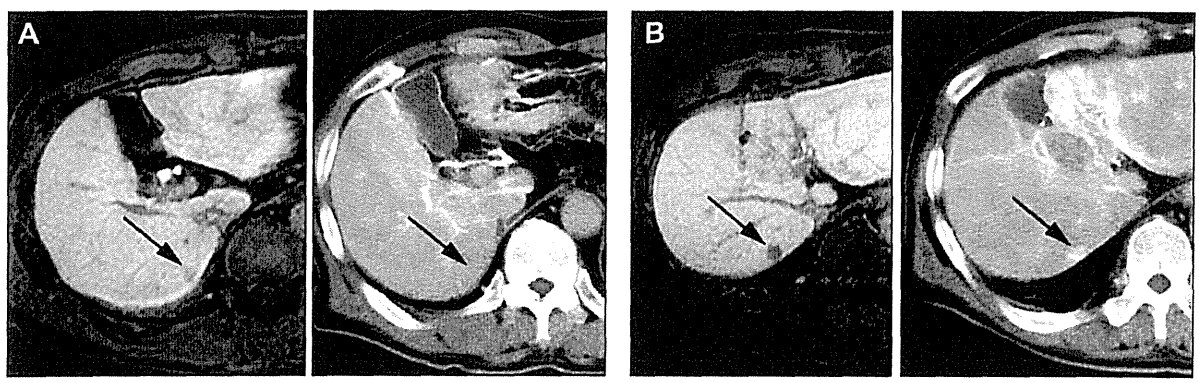

Fig. 3. Development of multicentric hepatocellular carcinoma in patients with preoperative non-hypervascular hypointense hepatic nodules detected during the hepatobiliary phase of Gd-EOB-DTPA-enhanced MRI. (A) Hepatobiliary phase of Gd-EOB-DTPA-enhanced MRI (left panel) and computed tomography during hepatic arteriography (CTHA, right panel) before hepatectomy for hepatocellular carcinoma (HCC). In addition to the typical HCC located in segment VIII, a hypointense hepatic nodule was detected in segment VI during the hepatobiliary phase of Gd-EOB-DTPA-enhanced MRI (arrow). No hypervascular nodule was detected at this site by CTHA (arrow). (B) Hepatobiliary phase of Gd-EOB-DTPA-enhanced MRI (left panel) and CTHA (right panel) 10 months after hepatectomy for HCC. The nodule detected during the hepatobiliary phase of Gd-EOB-DTPA-enhanced MRI showed minute growth in size, with clearer margin, compared to preoperative image (arrow). The hypervascularity of this nodule was identified by CTHA (arrow). This nodule was resected by re-hepatectomy and was diagnosed as HCC pathologically.

hypointense hepatic nodules observed preoperatively with Gd-EOB-DTPA-enhanced MRI. It is controversial whether all non-hypervascular hypointense nodules detected during the hepatobiliary phase of Gd-EOB-DTPA-enhanced MRI have the potential to progress to typical, hypervascular HCC. However, 26.5% of non-hypervascular hypointense nodules showed hypervascular spots with a long-term follow-up in our previous study [28]. In addition to the likelihood of non-hypervascular hypointense nodules

Please cite this article in press as: Toyoda H et al. Non-hypervascular hypointense nodules detected by Gd-EOB-DTPA-enhanced MRI are a risk factor for recurrence of HCC after hepatectomy. J Hepatol (2013), http://dx.doi.org/10.1016/j.jhep.2013.01.030

progressing to HCC, the results of the present study suggest that the presence of non-hypervascular hypointense nodules detected during the hepatobiliary phase of preoperative Gd-EOB-DTPA-enhanced MRI may indicate a high risk of multicentric recurrence of HCC after hepatectomy. Interestingly, multicentric recurrence was observed in all patients with multiple preoperative non-hypervascular hypointense nodules. While intrahepatic metastasis recurrence is considered as occurrence of metastasis of HCC that had been resected, multicentric recurrence is considered as new development of HCC that is not related to the resected HCC. Therefore, the presence of non-hypervascular hypointense nodules, especially multiple nodules, may indicate enhanced hepatocarcinogenesis even when the nodule itself does not progress to HCC.

There are several limitations to this study. The sample size was not large and the observation period was relatively short because Gd-EOB-DTPA has been in clinical use since February 2008 in Japan. In addition, the impact of the presence of non-hypervascular hypointense hepatic nodules on survival after hepatectomy was not analyzed because there were no patient deaths during the study period. However, we believe that our data should be shared with clinicians because of the markedly high rates of recurrence after hepatectomy in patients with preoperative non-hypervascular hypointense hepatic nodules. Further studies with more patients and a longer observation period are needed to confirm this observation. Furthermore, measures to suppress multicentric recurrence in patients with preoperative concurrent non-hypervascular hypointense hepatic nodules should be investigated in the future.

In conclusion, patients with preoperative concurrent non-hypervascular hypointense hepatic nodules, on the hepatobiliary phase of Gd-EOB-DTPA-enhanced MRI, are at higher risk of HCC recurrence after hepatectomy. Clinicians should take this into consideration when determining the treatment modalities.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

References

[1] Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74–108.
[2] Befeler AS, DiBisceglie AM. Hepatocellular carcinoma: diagnosis and treatment. Gastroenterology 2002;122:1609–1619.
[3] Umemura T, Kiyosawa K. Epidemiology of hepatocellular carcinoma in Japan. Hepatol Res 2007;37:S95–100.
[4] Kawata S, Murakami T, Kim T, Hori M, Federle MP, Kumano S, et al. Multidetector CT: diagnostic impact of slice thickness on detection of hypervascular hepatocellular carcinoma. AJR Am J Roentgenol 2002; 179:61–66.
[5] Ichikawa T, Erturk SM, Araki T. Multiphasic contrast-enhanced multidetector-row CT of liver: contrast-enhancement theory and practical scan protocol with a combination of fixed injection duration and patients' body-weight-tailored dose of contrast material. Eur J Radiol 2006; 58:165–176.
[6] Oka H, Kurioka N, Kim K, Kanno T, Kuroki T, Mizoguchi Y, et al. Prospective study of early detection of hepatocellular carcinoma in patients with cirrhosis. Hepatology 1990;12:680–687.
[7] Hamm B, Staks T, Muhler A, Bollow M, Taupitz M, Frenzel T, et al. Phase I clinical evaluation of Gd-EOB-DTPA as a hepatobiliary MR contrast agent: safety, pharmacokinetics, and MR imaging. Radiology 1995;195:785–792.
[8] Vogl TJ, Kummel S, Hammerstingl R, Schellenbeck M, Schumacher G, Balzer T, et al. Liver tumors: comparison of MR imaging with Gd-EOB-DTPA and Gd-DTPA. Radiology 1996;200:59–67.
[9] Kim SH, Kim SH, Lee J, Kim MJ, Jeon YH, Park Y, et al. Gadoxetic acid-enhanced MRI versus triple-phase MDCT for the preoperative detection of hepatocellular carcinoma. AJR Am J Roentgenol 2009;192:1675–1681.
[10] Van Beers BE, Pastor CM, Hussain HK. Primovist, eovist: what to expect? J Hepatol 2012;57:421–429.
[11] Reimer P, Rummeny EJ, Shamsi K, Balzer T, Daldrup HE, Tombach B, et al. Phase II clinical evaluation of Gd-EOB-DTPA: dose, safety aspects, and pulse sequence. Radiology 1996;199:177–183.
[12] Bruix J, Sherman M. Management of hepatocellular carcinoma. Hepatology 2005;42:1208–1236.
[13] Bruix J, Sherman M. Management of hepatocellular carcinoma: an update. Hepatology 2011;53:1020–1022.
[14] Kokudo N, Makuuchi M. Evidence-based clinical practice guidelines for hepatocellular carcinoma in Japan: J-HCC guidelines. J Gastroenterol 2009;44:S119–S121.
[15] Liver Cancer Study of Japan. Intrahepatic metastasis and multicentric occurrence. General rules for the clinical and pathological study of primary liver cancer. 3rd English ed. Tokyo: Kanehara Co. Ltd.; 2011, p. 54–55.
[16] Kumada T, Nakano S, Takeda I, Sugiyama K, Osada T, Kiriyama S, et al. Patterns of recurrence after initial treatment in patients with small hepatocellular carcinoma. Hepatology 1997;25:87–92.
[17] Tsuda H, Hirohashi S, Shimosato Y, Terada M, Hasegawa H. Clonal origin of atypical adenomatous hyperplasia of the liver and clonal identify with hepatocellular carcinoma. Gastroenterology 1988;95:1664–1666.
[18] Takenaka K, Adachi E, Nishizaki T, Hiroshige K, Ikeda T, Tsuneyoshi M, et al. Possible multicentric occurrence of hepatocellular carcinoma: a clinicopathological study. Hepatology 1994;19:889–894.
[19] Frericks BB, Loddenkemper C, Huppertz A, Valdeig S, Stroux A, Seja M, et al. Qualitative and quantitative evaluation of hepatocellular carcinoma and cirrhotic liver enhancement using Gd-EOB-DTPA. AJR Am J Roentgenol 2009;193:1053–1060.
[20] Matsui O, Kadoya M, Kameyama T, Yoshikawa J, Takashima T, Nakanuma Y, et al. Benign and malignant nodules in cirrhotic livers: distinction based on blood supply. Radiology 1991;178:493–497.
[21] Takayasu K, Muramatsu Y, Furukawa H, Wakao F, Moriyama N, Takayama T, et al. Early hepatocellular carcinoma: appearance at CT during arterial portography and CT arteriography with pathologic correlation. Radiology 1995;194:101–105.
[22] Hayashi M, Matsui O, Ueda K, Kawamori Y, Gabata T, Kadoya M. Progression to hypervascular hepatocellular carcinoma: correlation with intranodular blood supply evaluated with CT during intraarterial injection of contrast material. Radiology 2002;225:143–149.
[23] Kaplan EL, Meier P. Non parametric estimation for incomplete observation. J Am Stat Assoc 1958;53:457–481.
[24] Petro R, Pike MC. Conservation of the approximation (0–E2)/E in the log rank test for survival data on tumor incidence data. Biometrics 1973;29:579–584.
[25] Cox D. Regression models and life tables. J R Stat Soc 1972;34:187–220.
[26] Sano K, Ichikawa T, Motosugi U, Sou H, Muhi AM, Matsuda M, et al. Imaging study of early hepatocellular carcinoma: usefulness of gadoxetic acid-enhanced MR imaging. Radiology 2011;261:834–844.
[27] Kogita S, Imai Y, Okada M, Kim T, Onishi H, Takamura M, et al. Gd-EOB-DTPA-enhanced magnetic resonance images of hepatocellular carcinoma: correlation with histological grading and portal blood flow. Eur Radiol 2010;20:2405–2413.
[28] Kumada T, Toyoda H, Tada T, Sone Y, Fujimori M, Ogawa S, et al. Evolution of hypointense hepatocellular nodules observed only in the hepatobiliary phase using Gd-EOB-DTPA enhanced magnetic resonance imaging. AJR Am J Roentogenol 2011;197:58–63.

Please cite this article in press as: Toyoda H et al. Non-hypervascular hypointense nodules detected by Gd-EOB-DTPA-enhanced MRI are a risk factor for recurrence of HCC after hepatectomy. J Hepatol (2013), http://dx.doi.org/10.1016/j.jhep.2013.01.030

Journal of Medical Virology 85:228–234 (2013)

Comparison of the Efficacy of Ribavirin Plus Peginterferon Alfa-2b for Chronic Hepatitis C Infection in Patients With and Without Coagulation Disorders

Takashi Honda,[1] Yoshiaki Katano,[1]* Teiji Kuzuya,[1] Kazuhiko Hayashi,[1] Masatoshi Ishigami,[1] Akihiro Itoh,[1] Yoshiki Hirooka,[1] Isao Nakano,[1] Tetsuya Ishikawa,[1] Hidenori Toyoda,[2] Takashi Kumada,[2] Koji Yamamoto,[3] Tadashi Matsushita,[3] Tetsuhito Kojima,[3] Junki Takamatsu,[4] and Hidemi Goto[1]

[1]*Department of Gastroenterology and Hepatology, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[2]*Department of Gastroenterology, Ogaki Municipal Hospital, Ogaki, Gifu, Japan*
[3]*Department of Transfusion Medicine, Nagoya University Hospital, Nagoya, Japan*
[4]*Aichi Blood Center Japanese Red Cross, Seto, Japan*

Many patients with coagulation disorders are infected with hepatitis C virus (HCV) that advances to end stage liver disease, resulting in an increased number of deaths. The efficacy of ribavirin and peginterferon combination therapy for chronic HCV infection in patients with coagulation disorders has not been clarified fully. The aim of this study was to evaluate the efficacy and tolerability of combination therapy in this patient population compared with patients who are infected with HCV and do not have coagulation disorders. A total of 226 consecutive chronic hepatitis C patients were treated with combination therapy and divided into two groups: patients with (n = 23) and without coagulation disorders (n = 203). Clinical characteristics, sustained virological response rates obtained by an intention-to-treat analysis, and combination therapy discontinuation rates were compared between the two groups. The sustained virological response rates did not differ significantly between patients with and without coagulation disorders (65.2% vs. 47.8% by intention-to-treat analysis). According to a multivariate analysis, age, alanine aminotransferase, gamma-glutamyltransferase, and HCV genotype were associated significantly with a sustained virological response, whereas whether a patient had a coagulation disorder did not affect the sustained virological response. In conclusion, combination therapy for chronic hepatitis C was comparably effective between patients with and without coagulation disorders and did not result in adverse bleeding. ***J. Med. Virol. 85:228–234, 2013.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: chronic hepatitis C; interferon; ribavirin; coagulation disorders; hemophilia

INTRODUCTION

Hepatitis C virus (HCV) infection is a widespread viral infection that often leads to chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Until the 1980s, most patients with coagulation disorders became infected with HCV because of the extensive use of untreated factor concentrate. Some of these patients were infected with both hepatitis C and human immunodeficiency virus (HIV) [Brettler et al., 1990; Troisi et al., 1993; Yee et al., 2000; Franchini et al., 2001]. These patients with liver diseases and persistent abnormal transaminase progress to end stage liver disease, resulting in an increased number of liver disease-related deaths. In cases of co-infection with the HIV, the progression of liver disease is more rapid [Sanchez-Quijano et al., 1995; Soto et al., 1997; Benhamou et al., 1999; Ragni and Belle, 2001; De Luca et al., 2002] with a higher mortality rate than

Grant sponsor: Health Sciences Research Grants from the Ministry of Health, Labour and Welfare of Japan.

*Correspondence to: Yoshiaki Katano, Department of Gastroenterology and Hepatology, Nagoya University Graduate School of Medicine, 65 Tsuruma-Cho, Showa-Ku, Nagoya 466-8550, Japan E-mail: ykatano@med.nagoya-u.ac.jp

Accepted 10 September 2012

DOI 10.1002/jmv.23444

Published online 14 November 2012 in Wiley Online Library (wileyonlinelibrary.com).

during HCV monoinfection [Darby et al., 1997; Yee et al., 2000]. The need for treating infection with HCV in patients with coagulation disorders is increasing worldwide.

Sustained virological responders who are negative for serum HCV RNA 6 months after the end of treatment with interferon (IFN) are likely to remain in virological and biochemical remission with histologic improvement [Marcellin et al., 1997; Shiratori et al., 2000]. In addition, IFN therapy reduces the risk of hepatocellular carcinoma among virological or biochemical responders [Imai et al., 1998; Ikeda et al., 1999; Yoshida et al., 1999]. Ribavirin is now used generally in combination with IFN or pegIFN to treat chronic hepatitis C and combination therapy is more effective than IFN monotherapy [Lai et al., 1996; McHutchison et al., 1998; Poynard et al., 1998; Manns et al., 2001].

Previous studies have investigated the efficacy of IFN monotherapy in patients with coagulation disorders and chronic hepatitis C [Makris et al., 1991], and the efficacy of combination therapy with ribavirin and PegIFN in patients with coagulation disorders [Fried et al., 2002a; Mancuso et al., 2006; Posthouwer et al., 2007]. However, there are no reported comparisons of this combination therapy between patients infected with HCV with and without coagulation disorders. In this study, the efficacy and tolerability of ribavirin plus pegIFN were evaluated retrospectively in patients with coagulation disorders and chronic hepatitis C and the results were compared with the responses of patients infected with HCV but without coagulation disorders.

MATERIALS AND METHODS

Patients and Methods

A total of 226 consecutive patients with chronic hepatitis C and a high viral load (serum HCV RNA levels greater than 100 kilo-international units [KIU]) were treated with a combination of pegIFN and ribavirin between December 2004 and March 2007 at Nagoya University Hospital and Ogaki Municipal Hospital. These patients included 23 patients with coagulation disorders (17 with hemophilia A, 4 with hemophilia B, and 2 with von Willebrand disease). All patients were under 75 years old, were anti-HCV antibody-positive, and had serum HCV RNA levels greater than 100 KIU/ml by quantitative PCR assay (Amplicor GT-HCV Monitor Version 2.0; Roche Molecular Systems, Pleasanton, CA) within 12 weeks preceding the therapeutic period. Patients were excluded if they had pretreatment hemoglobin (Hb) levels <10 g/dl, tested positive for serum hepatitis B surface antigen, a history of drug addiction, alcohol abuse, autoimmune hepatitis, primary biliary cirrhosis, a serious psychiatric or medical illness, or were pregnant. To exclude patient bias, only complete cohorts from each hospital were enrolled. HCV genotypes were determined by PCR using genotype-specific primers [Okamoto et al., 1994; Simmonds et al., 1994].

All patients were treated with 1.5 μg/kg of pegIFN α-2b (Peg-Intron®, MSD, Tokyo, Japan) once weekly for 24 weeks in patients infected with HCV genotype 2 or 3 and for 48 weeks in patients infected with HCV genotype 1 or 4. For the 17 patients infected with HCV genotype 1, the treatment duration was extended to 72 weeks because of higher efficacy compared to that obtained after 48 weeks of treatment, but only in cases in which HCV RNA was positive at 12 weeks and negative at 24 weeks from the start of therapy. Treatment was discontinued when a patient's Hb concentration fell below 8.5 g/dl because of drug-induced hemolytic anemia or when a patient's white blood cell count fell below 1,000/mm^3, neutrophil count fell below 500/mm^3, or platelet count fell below 50,000/mm^3. Some patients discontinued treatment because the virus could not be eradicated after 24 weeks, as determined by the physician. The pegIFN alfa-2b dose was reduced to 50% of the assigned dose when the white blood cell count was below 1,500/mm^3, the neutrophil count below 750/mm^3 or the platelet count below 8,000/mm^3. Oral ribavirin (Rebetol®, MSD, Tokyo, Japan) was administered for the same duration as pegIFN at 600 mg/day for patients who weighed 60 kg or less, 800 mg/day for those who weighed more than 60 kg but less than 80 kg, and 1,000 mg/day for those who weighed more than 80 kg during the treatment period. The ribavirin dose was reduced by 200 mg/day when the patient's Hb concentration fell below 10 g/dl because of drug-associated hemolytic anemia. Ribavirin was discontinued when pegIFN therapy was discontinued. Informed consent was obtained from each patient and the study was performed in accordance with the 1975 Declaration of Helsinki.

Liver Histology

Pretreatment liver biopsy specimens were classified based on a fibrosis scale of F0 to F4 (F0, no fibrosis; F1, portal fibrosis without septa; F2, few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis) and in terms of necroinflammatory activity on a scale of A0 to A3 (A0, no histological activity; A1, mild activity; A2, moderate activity; and A3, severe activity) [Bedossa and Poynard, 1996; Fried et al., 2002b]. In patients with coagulation disorders, a liver biopsy was performed using factor concentrate, provided the patients gave informed consent.

Assessment of Efficacy

The virological response was assessed by a qualitative HCV RNA assay with a lower sensitivity limit of 100 copies/ml (Amplicor HCV version 2.0; Roche Molecular Systems). According to the qualitative HCV RNA results, responses were defined as a sustained virological response if no HCV RNA was detected at the end of the 24-week follow-up period after the treatment was completed. A patient was considered to have an end of treatment virological response if no HCV RNA was detected at the end of treatment.

Comparison of Characteristics and Treatment Efficacy Between Patients With and Without Coagulation Disorders

Sex ratio, age, body weight, body mass index (BMI), baseline serum alanine aminotransferase (ALT) levels, gamma-glutamyltransferase (GGT), pretreatment Hb level, platelet counts, HCV genotype and viral load, histologic activity, and fibrosis were compared between patients with and without coagulation disorders. The sustained virological response rates obtained by an intention-to-treat analysis and per-protocol analysis, ribavirin and pegIFN dose reduction rates, and combination therapy discontinuation rates were compared between the two groups. The end of treatment virological response rate was obtained by intention-to-treat and per-protocol analyses and then compared between the two groups. Next, the variable accession method in a multivariate analysis was used to examine factors associated with a sustained virological response after combination therapy, including the following factors: sex, age, BMI, baseline serum ALT, GGT, platelet counts, genotype, HCV RNA concentration, and presence of a coagulation disorder.

Because efficacy differed by the HCV genotype and the patient age, and since all coagulation disorder patients were male, the analysis focused on male, age-matched patients infected with HCV genotype 1. The characteristics and efficacy of treatment were compared in males, and age-matched patients with and without coagulation disorders who were infected with HCV-genotype 1.

Statistical Analysis

Values are expressed as the means ± SDs. Between-group differences in mean quantitative values were analyzed by Student's *t*-test, and differences in nonparametric data were analyzed by the Mann–Whitney *U*-test. Differences in proportions were examined by the Chi-squared test. Multiple logistic regression analysis was used to identify factors related to a sustained virological response. All statistical analyses were performed using SAS software (SAS Institute, Cary, NC). All P values were two-tailed, and $P < 0.05$ was considered statistically significant.

RESULTS

Patient Characteristics

The patients included 127 men and 99 women aged 22–74 years (mean ± SD, 54.7 ± 11.6). The mean age of patients without coagulation disorders was 56.3 ± 10.9 years and most patients were in their 50s and 60s. In contrast, the mean age of patients with coagulation disorders was 41.5 ± 9.8 years with an age distribution ranging from 20 to 50 years. The clinical characteristics of the two study groups are shown in Table I. All patients with coagulation disorders in this study were male because of inherited, sex-linked hemophilia, and two patients in this study had male von Willebrand disease. Patients with coagulation disorders were significantly younger than patients without coagulation disorders ($P < 0.0001$). Although body weight was not different between the two groups, patients with coagulation disorders had a significantly lower BMI than patients without coagulation disorders. Patients without coagulation disorders were infected with HCV genotypes that are not unique to Japan, such as genotypes 1a, 3a, and 4a. Four patients with coagulation disorders were infected with human immunodeficiency virus and one of these patients had achieved a sustained virological response.

Response to Therapy

The ribavirin dose reduction rate tended to be higher in patients without coagulation disorders than in patients with coagulation disorders ($P = 0.0643$). The treatment discontinuation rate did not differ significantly between the two groups. As a result, the sustained virological response rate by an intention-to-treat analysis did not differ significantly between the

TABLE I. Clinical Characteristics of Patients Treated With Combination Therapy

	Total patients (n = 226)	Patients without coagulation disorders (n = 203)	Patients with coagulation disorders (n = 23)	*P* value
Sex ratio (male/female)	127/99	104/99	23/0	<0.0001
Age (years)	54.7 ± 11.6	56.3 ± 10.9	41.5 ± 9.8	<0.0001
Body weight (kg)	60.2 ± 11.1	60.5 ± 11.5	60.5 ± 8.1	0.9972
Body mass index	22.9 ± 3.1	23.1 ± 3.1	21.5 ± 2.5	0.0226
Baseline serum ALT (IU/L)	63.3 ± 56.8	60.9 ± 54.9	84.4 ± 69.1	0.0598
GGT (IU/L)	54.2 ± 63.9	51.4 ± 62.2	78.6 ± 74.4	0.0526
Hemoglobin (g/dl)	14.1 ± 1.3	14.1 ± 1.3	14.4 ± 1.3	0.2714
Platelets ($\times 10^4/\mu l$)	17.8 ± 5.2	17.7 ± 5.2	19.0 ± 5.6	0.2597
Genotype (1a/1b/2a/2b/3a/4a)	7/160/40/15/3/1	0/150/39/14/0/0	7/10/1/1/3/1	<0.0001
HCV RNA (KIU/ml)	1,898.0 ± 1,448.3	1,923.1 ± 1,464.5	1,676.6 ± 1,305.1	0.4404
Activity (A0/A1/A2/A3)	2/108/71/11	2/101/64/11	0/7/7/0	0.3442
Fibrosis (F0/F1/F2/F3)	17/104/49/22	16/97/45/20	1/7/4/2	0.5351

ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; HCV RNA, hepatitis C virus RNA; KIU, kilo-international units.

TABLE II. Efficacy of Combination Therapy

	Total patients (n = 226)	Patients without coagulation disorders (n = 203)	Patients with coagulation disorders (n = 23)	*P* value
SVR rate (intention-to-treat)	49.6 (112/226)	47.8 (97/203)	65.2 (15/23)	0.1130
SVR rate (per-protocol)	54.4 (111/204)	52.7 (97/184)	70.0 (14/20)	0.1405
ETR rate (intention-to-treat)	84.1 (190/226)	84.7 (172/203)	78.3(18/23)	0.4218
ETR rate (per-protocol)	89.1 (179/201)	89.6 (163/182)	84.2 (16/19)	0.4772
Ribavirin dose reduction rate	44.2 (100/226)	46.3 (94/203)	26.1 (6/23)	0.0643
PegIFN dose reduction rate	34.1 (77/226)	33.5 (68/203)	39.1 (9/23)	0.5891
Combination therapy discontinuation rate	9.8 (22/226)	9.4 (19/203)	13.0 (3/23)	0.5722

SVR, sustained virological response; ETR, end of treatment virological response; PegIFN, peginterferon.

two groups. The sustained virological response rate of patients with coagulation disorders by a per-protocol analysis was higher than that of patients without coagulation disorders, but there was no significant difference. In addition, based on both intention-to-treat and per-protocol analyses, the end of treatment virological response rate did not differ significantly between the two groups (Table II).

Factors associated with a sustained virological response in combination therapy were determined by a multivariate analysis. HCV genotype 1 and 4 versus 2 and 3 ($P = 0.001$, odds ratio 4.353 [95% CI, 1.810–10.469]), baseline serum GGT ($P = 0.003$, odds ratio 1.018 [1.006–1.030]), age ($P = 0.006$, odds ratio 1.053 [1.015–1.093]), and baseline serum ALT ($P = 0.014$, odds ratio 0.991 [0.983–0.998]) were associated significantly with a sustained virological response, but whether or not a patient had a coagulation disorder was not associated significantly with a sustained virological response.

Characteristics and Response of Male, Age-Matched Patients Infected With HCV Genotype 1

The clinical characteristics of the two study groups in the male, age-matched patients infected with HCV genotype 1 are shown in Table III. Body weight, BMI, and Hb levels were significantly lower in patients with coagulation disorders than patients without coagulation disorders ($P = 0.0003$, 0.0027, and 0.0103, respectively).

The treatment discontinuation rate of patients with coagulation disorders did not differ between the two groups. The sustained virological response rate by intention-to-treat and per-protocol analyses did not differ significantly between the two groups (Table IV). Factors associated with a sustained virological response in the male, age-matched, genotype 1 patients treated with combination therapy were determined by a multivariate analysis. BMI ($P = 0.036$, odds ratio 1.810 [1.041–3.145]) and baseline serum GGT ($P = 0.037$, odds ratio 0.981 [0.963–0.999]) were associated significantly with a sustained virological response, but whether or not a patient had a coagulation disorder was not associated significantly with a sustained virological response.

Adverse Events

The reasons for discontinuing combination therapy and the times at which the therapy was discontinued are shown in Table V. Once treatment was discontinued, therapy was not restarted even after the initial symptoms or illness disappeared. There were no bleeding episodes in the patients with coagulation disorders, including patients who received a liver biopsy.

TABLE III. Clinical Characteristics of Male, Age-Matched Patients With Genotype 1 Treated With Combination Therapy

	Total patients (n = 36)	Patients without coagulation disorders (n = 18)	Patients with coagulation disorders (n = 18)	*P* value
Age (years)	42.8 ± 8.0	44.9 ± 5.9	40.7 ± 9.3	0.1136
Body weight (kg)	66.1 ± 11.0	73.4 ± 9.3	60.4 ± 8.7	0.0003
Body mass index	22.7 ± 2.8	24.3 ± 2.3	21.4 ± 2.5	0.0027
Baseline serum ALT (IU/L)	69.8 ± 54.3	63.5 ± 31.7	76.2 ± 70.5	0.4919
GGT (IU/L)	72.7 ± 64.2	74.3 ± 71.1	71.2 ± 58.5	0.8869
Hemoglobin (g/dl)	14.9 ± 1.2	15.4 ± 1.0	14.4 ± 1.2	0.0103
Platelets ($\times 10^4/\mu$l)	19.3 ± 5.4	18.8 ± 4.5	19.8 ± 5.6	0.5773
HCV RNA (KIU/ml)	2,050.8 ± 1,273.4	2,322.8 ± 1,249.1	1,778.8 ± 1,273.5	0.2044
Activity (A0/A1/A2/A3)	0/12/11/0	0/6/5/0	0/6/6/0	0.6723
Fibrosis (F0/F1/F2/F3)	2/11/8/2	1/5/4/1	1/6/4/1	0.9392

ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; HCV RNA, hepatitis C virus RNA; KIU, kilo-international unit.

TABLE IV. Efficacy of Combination Therapy in Male, Age-Matched Patients With Genotype 1

	Total patients (n = 36)	Patients without coagulation disorders (n = 18)	Patients with coagulation disorders (n = 18)	*P* value
SVR rate (intention-to-treat)	58.3 (21/36)	61.1 (11/18)	55.6 (10/18)	0.7353
SVR rate (per-protocol)	69.0 (20/29)	64.7 (11/17)	75.0 (9/12)	0.5551
ETR rate (intention-to-treat)	77.8 (28/36)	83.3 (15/18)	72.2 (13/18)	0.4227
ETR rate (per-protocol)	93.1 (27/29)	88.2 (15/17)	100.0 (12/12)	0.2182
Ribavirin dose reduction rate	22.2 (28/36)	16.7 (3/18)	27.8 (5/18)	0.7175
PegIFN dose reduction rate	36.1 (13/36)	27.8 (5/18)	44.4 (8/18)	0.2979
Combination therapy discontinuation rate	5.6 (2/36)	0 (0/18)	16.7 (3/18)	0.0704

SVR, sustained virological response; ETR, end of treatment virological response; PegIFN, peginterferon.

DISCUSSION

A previous randomized trial in patients infected with HCV with inherited bleeding disorders showed that the sustained virological response rate improved significantly for patients who were treated with IFN and ribavirin compared to those treated with IFN alone [Fried et al., 2002a]. In addition, both chronic hepatitis C patients with and without coagulation disorders responded similarly to pegIFN and ribavirin combination therapy [Franchini et al., 2006; Posthouwer et al., 2006]. However, the efficacy and tolerability of this combination therapy differed based on the HCV genotype as well as the age, gender, and race of the patients; therefore it is difficult to compare patients with and without coagulation disorders under the same conditions. No report has examined that patients infected chronic hepatitis C with and without coagulation disorders at the same institution and during the same observation period. In addition, there are no reports on the efficacy of combination therapy in patients with chronic hepatitis C with and without coagulation disorders in age-matched patients infected with HCV genotype 1. Therefore, a retrospective study was conducted to evaluate the efficacy and tolerability of ribavirin plus pegIFN in chronic hepatitis C patients with and without coagulation disorders. In the per-protocol analysis, there were no significant differences, but the sustained virological response rate was higher in patients with coagulation disorders than in patients without coagulation disorders. Mancuso et al. [2006] reported that combination therapy with pegIFN alfa-2b plus ribavirin is highly efficacious in hemophiliacs with chronic hepatitis C. In an overall analysis, patients with coagulation disorders had a lower mean age than patients without coagulation disorders. In addition, the BMI of the patients with coagulation disorders was lower than that of patients without coagulation disorders. A multivariate analysis showed that the HCV genotype, baseline serum GGT, age, and baseline ALT were factors associated significantly with a sustained virological response and whether patients had coagulation disorders was not associated with a sustained virological response. Age, especially younger than 40 years old, was a good predictive factor for a sustained virological response, as was reported previously [Poynard et al., 2000; Fried et al., 2002b].

TABLE V. Reasons for Discontinuing Combination Therapy

Reason	Number	Weeks after starting treatment
Patients with coagulation disorders		
Peritonitis due to appendicitis	1	16
Pneumoniae	1	18
No HCV eradication	3	24, 28, 29
IDDM	1	44
Patients without coagulation disorders		
Fatigue	5	1, 2, 4.9, 19
Bleeding from duodenal varies	1	8
Dizziness	1	12
Palpitation	1	13
Cholecystitis	1	16
Symptom of Parkinson's disease	1	16
Fundal hemorrhage	1	17
Hepatocellular carcinoma	2	19, 21
Suspicion of Interstitial pneumonia	1	20
Gastric cancer	2	21, 36
Self-discontinuation	1	24
Neutropenia	1	25
Eruption	1	25
No HCV eradication	7	24, 25, 25, 27, 28, 29, 29

These results suggest that male patients who are infected with HCV genotype 1 and have coagulation disorders will have a higher sustained virological response than patients without coagulation disorders, if the coagulation disorder patients do not discontinue treatment. However, these results do not account for the differences in age. Therefore, male, age-matched patients infected with HCV genotype 1 were evaluated. The characteristics that differed between patients with and without coagulation disorders were body weight, BMI and baseline Hb levels.

In male, age-matched patients infected with HCV genotype 1, the sustained virological response rate based on both intention-to-treat and per-protocol analyses was not different between patients with and without coagulation disorders.

Using a multivariate analysis, whether patients had coagulation disorders was not associated significantly with a sustained virological response. Only BMI and GGT were identified as factors associated with a sustained virological response to combination therapy in male, age-matched patients infected with HCV genotype 1. A previous report showed that GGT levels may represent a surrogate marker of tumor necrosis factor-alpha expression in the liver and explain the importance of serum analyses to in predict the treatment outcome [Taliani et al., 2002]. Several studies revealed that GGT is one predictor of a sustained virological response [Taliani et al., 2002, 2006; Villela-Nogueira et al., 2005]. In western countries, obesity and a high BMI are associated with the absence of a sustained virological response to combination therapy of pegIFN or IFN with ribavirin [Bressler et al., 2003; Camma et al., 2004]. However, in Japan, most of the patients who are treated with combination therapy are not obese and have lower BMIs than patients in western countries. In this population, the mean BMI was 22.7 ± 2.8. In this low BMI population, a higher BMI would be associated with a sustained virological response. However, the reason why a low BMI is associated with the absence of a sustained virological response has not elucidated.

Adverse effects are thought to increase in patients with coagulation disorders; however, there was not a significant difference in adverse effects necessitating discontinuation of pegIFN and ribavirin between patients with and without coagulation disorders (13.0% vs. 9.4%). In addition, severe adverse effects and bleeding adverse effects were not associated with coagulation disorders. A previous report showed that IFN and ribavirin combination therapy may reduce the use of clotting factors in hemophilia patients with chronic hepatitis C [Honda et al., 2005; Yamamoto et al., 2006]. Ribavirin may reduce the side effect of bleeding during combination therapy. In this study, patients with coagulation disorders did not experience an adverse effect of bleeding.

In conclusion, treatment of chronic hepatitis C with combination therapy was effective comparably between patients with and without coagulation disorders and there were no adverse effects of bleeding.

REFERENCES

Bedossa P, Poynard T. 1996. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. Hepatology 24:289–293.

Benhamou Y, Bochet M, Di Martino V, Charlotte F, Azria F, Coutellier A, Vidaud M, Bricaire F, Opolon P, Katlama C, Poynard T. 1999. Liver fibrosis progression in human immunodeficiency virus and hepatitis C virus coinfected patients. The Multivirc Group. Hepatology 30:1054–1058.

Bressler BL, Guindi M, Tomlinson G, Heathcote J. 2003. High body mass index is an independent risk factor for nonresponse to antiviral treatment in chronic hepatitis C. Hepatology 38:639–644.

Brettler DB, Alter HJ, Dienstag JL, Forsberg AD, Levine PH. 1990. Prevalence of hepatitis C virus antibody in a cohort of hemophilia patients. Blood 76:254–256.

Camma C, Di Bona D, Schepis F, Heathcote EJ, Zeuzem S, Pockros PJ, Marcellin P, Balart L, Alberti A, Craxi A. 2004. Effect of peginterferon alfa-2a on liver histology in chronic hepatitis C: A meta-analysis of individual patient data. Hepatology 39:333–342.

Darby SC, Ewart DW, Giangrande PL, Spooner RJ, Rizza CR, Dusheiko GM, Lee CA, Ludlam CA, Preston FE. 1997. Mortality from liver cancer and liver disease in haemophilic men and boys in UK given blood products contaminated with hepatitis C. UK Haemophilia Centre Directors' Organisation. Lancet 350:1425–1431.

De Luca A, Bugarini R, Lepri AC, Puoti M, Girardi E, Antinori A, Poggio A, Pagano G, Tositti G, Cadeo G, Macor A, Toti M, D'Arminio Monforte A. 2002. Coinfection with hepatitis viruses and outcome of initial antiretroviral regimens in previously naive HIV-infected subjects. Arch Intern Med 162:2125–2132.

Franchini M, Rossetti G, Tagliaferri A, Capra F, de Maria E, Pattacini C, Lippi G, Lo Cascio G, de Gironcoli M, Gandini G. 2001. The natural history of chronic hepatitis C in a cohort of HIV-negative Italian patients with hereditary bleeding disorders. Blood 98:1836–1841.

Franchini M, Nicolini N, Capra F. 2006. Treatment of hepatitis C in hemophiliacs. Am J Hematol 81:696–702.

Fried MW, Peter J, Hoots K, Gaglio PJ, Talbut D, Davis PC, Key NS, White GC, Lindblad L, Rickles FR, Abshire TC. 2002a. Hepatitis C in adults and adolescents with hemophilia: A randomized, controlled trial of interferon alfa-2b and ribavirin. Hepatology 36:967–972.

Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. 2002b. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 347: 975–982.

Honda T, Toyoda H, Hayashi K, Katano Y, Yano M, Nakano I, Yoshioka K, Goto H, Yamamoto K, Takamatsu J. 2005. Ribavirin and use of clotting factors in patients with hemophilia and chronic hepatitis C. JAMA 293:1190–1192.

Ikeda K, Saitoh S, Arase Y, Chayama K, Suzuki Y, Kobayashi M, Tsubota A, Nakamura I, Murashima N, Kumada H, Kawanishi M. 1999. Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis type C: A long-term observation study of 1,643 patients using statistical bias correction with proportional hazard analysis. Hepatology 29:1124–1130.

Imai Y, Kawata S, Tamura S, Yabuuchi I, Noda S, Inada M, Maeda Y, Shirai Y, Fukuzaki T, Kaji I, Ishikawa H, Matsuda Y, Nishikawa M, Seki K, Matsuzawa Y. 1998. Relation of interferon therapy and hepatocellular carcinoma in patients with chronic hepatitis C. Osaka Hepatocellular Carcinoma Prevention Study Group. Ann Intern Med 129:94–99.

Lai MY, Kao JH, Yang PM, Wang JT, Chen PJ, Chan KW, Chu JS, Chen DS. 1996. Long-term efficacy of ribavirin plus interferon alfa in the treatment of chronic hepatitis C. Gastroenterology 111:1307–1312.

Makris M, Preston FE, Triger DR, Underwood JC, Westlake L, Adelman MI. 1991. A randomized controlled trial of recombinant interferon-alpha in chronic hepatitis C in hemophiliacs. Blood 78:1672–1677.

Mancuso ME, Rumi MG, Santagostino E, Linari S, Coppola A, Mannucci PM, Colombo M. 2006. High efficacy of combined therapy

with pegylated interferon plus ribavirin in patients with hemophilia and chronic hepatitis C. Haematologica 91:1367–1371.

Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: A randomised trial. Lancet 358:958–965.

Marcellin P, Boyer N, Gervais A, Martinot M, Pouteau M, Castelnau C, Kilani A, Areias J, Auperin A, Benhamou JP, Degott C, Erlinger S. 1997. Long-term histologic improvement and loss of detectable intrahepatic HCV RNA in patients with chronic hepatitis C and sustained response to interferon-alpha therapy. Ann Intern Med 127:875–881.

McHutchison JG, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, Goodman ZD, Ling MH, Cort S, Albrecht JK. 1998. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. N Engl J Med 339:1485–1492.

Okamoto H, Mishiro S, Tokita H, Tsuda F, Miyakawa Y, Mayumi M. 1994. Superinfection of chimpanzees carrying hepatitis C virus of genotype II/1b with that of genotype III/2a or I/1a. Hepatology 20:1131–1136.

Posthouwer D, Mauser-Bunschoten EP, Fischer K, Makris M. 2006. Treatment of chronic hepatitis C in patients with haemophilia: A review of the literature. Haemophilia 12:473–478.

Posthouwer D, Yee TT, Makris M, Fischer K, Griffioen A, Van Veen JJ, Mauser-Bunschoten EP. 2007. Antiviral therapy for chronic hepatitis C in patients with inherited bleeding disorders: An international, multicenter cohort study. J Thromb Haemost 5: 1624–1629.

Poynard T, Marcellin P, Lee SS, Niederau C, Minuk GS, Ideo G, Bain V, Heathcote J, Zeuzem S, Trepo C, Albrecht J. 1998. Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). Lancet 352:1426–1432.

Poynard T, McHutchison J, Goodman Z, Ling MH, Albrecht J. 2000. Is an "a la carte" combination interferon alfa-2b plus ribavirin regimen possible for the first line treatment in patients with chronic hepatitis C? The ALGOVIRC Project Group. Hepatology 31:211–218.

Ragni MV, Belle SH. 2001. Impact of human immunodeficiency virus infection on progression to end-stage liver disease in individuals with hemophilia and hepatitis C virus infection. J Infect Dis 183:1112–1115.

Sanchez-Quijano A, Andreu J, Gavilan F, Luque F, Abad MA, Soto B, Munoz J, Aznar JM, Leal M, Lissen E. 1995. Influence of human immunodeficiency virus type 1 infection on the natural course of chronic parenterally acquired hepatitis C. Eur J Clin Microbiol Infect Dis 14:949–953.

Shiratori Y, Imazeki F, Moriyama M, Yano M, Arakawa Y, Yokosuka O, Kuroki T, Nishiguchi S, Sata M, Yamada G, Fujiyama S, Yoshida H, Omata M. 2000. Histologic improvement of fibrosis in patients with hepatitis C who have sustained response to interferon therapy. Ann Intern Med 132:517–524.

Simmonds P, Alberti A, Alter HJ, Bonino F, Bradley DW, Brechot C, Brouwer JT, Chan SW, Chayama K, Chen DS, Choo QL, Colombo M, Cuypers HM, Date T, Dusheiko GM, Esteban JI, Fay O, Hadziyannis SJ, Han J, Hatzakis A, Holmes EC, Hotta H, Houghton M, Irvine B, Kohara M, Kolberg JA, Kuo G, Lau JN, Lelie PN, Maertens G, McOmish F, Miyamura T, Mizokami M, Nomoto A, Prince AM, Reesink HW, Rice C, Roggendorf M, Schalm SW, Shikata T, Shimotohno K, Stuyver L, Trépo C, Weiner A, Yap PL, Urdea MS. 1994. A proposed system for the nomenclature of hepatitis C viral genotypes. Hepatology 19: 1321–1324.

Soto B, Sanchez-Quijano A, Rodrigo L, del Olmo JA, Garcia-Bengoechea M, Hernandez-Quero J, Rey C, Abad MA, Rodriguez M, Sales Gilabert M, Gonzalez F, Miron P, Caruz A, Relimpio F, Torronteras R, Leal M, Lissen E. 1997. Human immunodeficiency virus infection modifies the natural history of chronic parenterally-acquired hepatitis C with an unusually rapid progression to cirrhosis. J Hepatol 26:1–5.

Taliani G, Badolato MC, Nigro G, Biasin M, Boddi V, Pasquazzi C, Clerici M. 2002. Serum concentration of gammaGT is a surrogate marker of hepatic TNF-alpha mRNA expression in chronic hepatitis C. Clin Immunol 105:279–285.

Taliani G, Gemignani G, Ferrari C, Aceti A, Bartolozzi D, Blanc PL, Capanni M, Esperti F, Forte P, Guadagnino V, Mari T, Marino N, Milani S, Pasquazzi C, Rosina F, Tacconi D, Toti M, Zignego AL, Messerini L, Stroffolini T. 2006. Pegylated interferon alfa-2b plus ribavirin in the retreatment of interferon-ribavirin nonresponder patients. Gastroenterology 130:1098–1106.

Troisi CL, Hollinger FB, Hoots WK, Contant C, Gill J, Ragni M, Parmley R, Sexauer C, Gomperts E, Buchanan G, Schwartz B, Adair S, Fields H. 1993. A multicenter study of viral hepatitis in a United States hemophilic population. Blood 81:412–418.

Villela-Nogueira CA, Perez RM, de Segadas Soares JA, Coelho HS. 2005. Gamma-glutamyl transferase (GGT) as an independent predictive factor of sustained virologic response in patients with hepatitis C treated with interferon-alpha and ribavirin. J Clin Gastroenterol 39:728–730.

Yamamoto K, Honda T, Matsushita T, Kojima T, Takamatsu J. 2006. Anti-HCV agent, ribavirin, elevates the activity of clotting factor VII in patients with hemophilia: A possible mechanism of decreased events of bleeding in patients with hemophilia by ribavirin. J Thromb Haemost 4:469–470.

Yee TT, Griffioen A, Sabin CA, Dusheiko G, Lee CA. 2000. The natural history of HCV in a cohort of haemophilic patients infected between 1961 and 1985. Gut 47:845–851.

Yoshida H, Shiratori Y, Moriyama M, Arakawa Y, Ide T, Sata M, Inoue O, Yano M, Tanaka M, Fujiyama S, Nishiguchi S, Kuroki T, Imazeki F, Yokosuka O, Kinoyama S, Yamada G, Omata M. 1999. Interferon therapy reduces 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 Intern Med 131:174–181.

Biomarkers, 2013; 18(1): 82–87

ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.3109/1354750X.2012.738249

informa
healthcare

RESEARCH ARTICLE

Higher hepatic gene expression and serum levels of matrix metalloproteinase-2 are associated with steatohepatitis in non-alcoholic fatty liver diseases

Hidenori Toyoda[1], Takashi Kumada[1], Seiki Kiriyama[1], Makoto Tanikawa[1], Yasuhiro Hisanaga[1], Akira Kanamori[1], Toshifumi Tada[1], and Yoshiki Murakami[2]

[1]*Department of Gastroenterology, Ogaki Municipal Hospital, Ogaki, Japan and* [2]*Center for Genomic Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan*

Abstract

We investigated the gene expression of tissue inhibitor metalloproteinases (TIMPs) and matrix metalloproteinases (MMPs) and serum levels of TIMPs, MMPs, and hyaluronic acid that are associated with liver fibrosis in 64 patients with nonalcoholic fatty liver diseases (NAFLD). Whereas, no differences were found between patients with and without nonalcoholic steatohepatitis (NASH) in serum levels of hyaluronic acid when excluding NASH patients with advanced fibrosis, the quantity of MMP2 mRNA in liver tissue and serum MMP2 levels were significantly higher in patients with NASH than those without, even focusing on patients with less advanced fibrosis, indicating the initiation of liver fibrosis.

Keywords: Non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, matrix metalloproteinases, tissue inhibitors of metalloproteinases, hyaluronic acid, gene expression, serum levels

Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common liver diseases in both Western and Asian countries (Angulo 2002; Clark et al. 2003; Kojima et al. 2003; Chitturi et al. 2007; Torres & Harrison 2008), affecting 30% of the general Western adult population (Musso et al. 2010). NAFLD encompasses a histological spectrum that ranges from simple steatosis to nonalcoholic steatohepatitis (NASH). Whereas simple steatosis is usually benign, patients with NASH can progress to cirrhosis and end-stage liver disease (Angulo 2002; Fassio et al. 2004; Hashimoto et al. 2005). Therefore, it is important to differentiate patients with NASH from patients with more benign forms of NAFLD.

Liver fibrosis accumulates with the progression of NASH toward cirrhosis, as is reported in the case of viral hepatitis. Changes in many proteins associated with fibrosis have been reported during the course of viral hepatitis. Matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of metalloproteinases, TIMPs) are reportedly associated with the progression of liver fibrosis (Hemmann et al. 2007). It is unclear whether changes in the gene expression of fibrosis-associated proteins occur in the liver of patients with NASH, as they do in patients with viral hepatitis, and whether there are differences in the gene expression patterns and serum levels of these proteins between NASH and simple steatosis. In the present study, we investigated the gene expression patterns of several fibrosis-associated proteins in the livers of patients with NAFLD, comparing patients with and without NASH. Serum levels of fibrosis-associated proteins were also investigated.

Patients and methods

Patients

The study population consisted of 64 patients (36 males and 28 females with a mean age of 51.0±15.0

Address for Correspondence: Hidenori Toyoda, MD, PhD, Department of Gastroenterology, Ogaki Municipal Hospital, 4–86, Minaminokawa, Ogaki, Gifu, 503–8502, Japan. Tel: +81-584-81-3341. Fax: +81-584-75-5715. E-mail: tkumada@he.mirai.ne.jp

(Received 13 August 2012; revised 24 September 2012; accepted 05 October 2012)

years) who underwent ultrasound-guided liver biopsy between 2008 and 2010 for the diagnosis of NAFLD. They were patients who agreed with liver biopsy among 268 patients who had admitted to our clinics and had been advised to receive liver biopsy because of the clinical diagnosis of NAFLD during the study period. Liver biopsy was performed to examine the presence of NASH and to confirm the diagnosis. Patients were clinically diagnosed with NAFLD prior to biopsy based on the following criteria: (i) persistent abnormal liver function tests for more than 3 months, (ii) ultrasonographic images showing steatosis, (iii) no evidence of alcohol abuse, and (iv) exclusion of other liver diseases and other known causes of steatosis based on the results of specific clinical, biochemical, or imaging studies. The ultrasonographic findings of steatosis were based on established criteria such as hepatorenal echo contrast, liver brightness, deep attenuation, and vascular blurring (Hamaguchi et al. 2007). The first two criteria were used as definitive criteria, while the latter two criteria were taken into account as needed. All patients were confirmed not to have chronic viral hepatitis with negative results for hepatitis B virus (HBV) surface antigen, HBV DNA, hepatitis C virus (HCV) antibody, and HCV RNA. No patients were diagnosed as having autoimmune hepatitis, primary biliary cirrhosis, or other liver diseases.

All patients underwent ultrasonography-guided fine needle liver biopsy using a 17G biopsy needle. NAFLD was pathologically diagnosed based on pathologic findings in the biopsied liver specimens. The liver biopsy specimens were stained with hematoxylin and eosin, Masson's trichrome, and periodic-acid Schiff stains and then examined by experienced pathologists. The liver specimens were categorized into types 1–4 pathologically based on Matteoni classification (Matteoni et al. 1999), and types 3 and 4 were defined as NASH. Patologic evaluations were performed by two pathologists independently.

The study protocol was in compliance with the Helsinki Declaration and was approved by the institutional review board of Ogaki Municipal Hospital. All patients provided written informed consent for the use of their clinical data and the analyses of biopsy specimens and serum samples.

RNA extraction and real-time PCR for gene expression analyses

Liver biopsy specimens were stored in Ambion RNAlater solution (Life Technologies, Carlsbad, CA, USA) at –80°C until RNA extraction. Total RNA was extracted using the mirVana miRNA isolatidon kit (Life Technologies) according to the manufacturer's instructions.

cDNA was synthesized using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Basel, Switzerland). Total RNA (2 mg) in 10.4 μL of nuclease-free water was added to 1 mL of 50 mM random hexamer. The denaturing reaction was performed for 10 min at 65°C. The denatured RNA mixture was added to 4 mL of 5× reverse transcriptase buffer, 2 mL of 10 mM dNTP, 0.5 mL of 40 U/mL RNase inhibitor, and 1.1 mL of reverse transcriptase (FastStart Universal SYBR Green Master, Roche) in a total volume of 20 mL. The reaction ran for 30 min at 50°C (cDNA synthesis), and 5 min at 85°C (enzyme denaturation). All reactions were run in triplicate. The Chromo4 detector (Bio-Rad, Hercules, CA, USA) was used to detect mRNA expression. The primer sequences are follows: TIMP1: 5′-cttggcttctgcactgatgg-3′ (sense), 5′-acgctggtataaggtggtct-3′ (antisense); TIMP2: 5′-agtggactctggaaacgaca-3′ (sense); 5′-tctctgtgacccagtccatc-3′ (antisense); MMP2: 5′- aacgccgatggggagtactg-3′ (sense); 5′-cagggctgtccttcagcgtt-3′ (antisense); MMP13: 5′-gaggctccgagaaatgcagt-3′ (sense); 5′-atgccatcgtgaagtctggt-3′ (antisense); and β-actin: 5′-ccactggcatcgtgatggac-3′ (sense), 5′-tcattgccaatggtgatgacct-3′ (antisense). Assays were performed in triplicate, and the expression levels of target genes were normalized to the expression of the β-actin gene as quantified using real-time quantitative PCR as an internal control.

Measurement of serum levels of fibrosis-associated proteins

Serum levels of TIMP1, MMP2, and hyaluronic acid were measured in stored fasting serum samples that had been obtained at the time of liver biopsy. Serum TIMP1 levels were measured by enzyme immunoassay (hTIMP-1 kit, Daiichi Fine Chemical, Toyama, Japan). Serum MMP2 levels were measured by enzyme immunoassay (hMMP-2 Activity Assay System, GE Healthcare Japan, Tokyo, Japan). Serum hyaluronic acid was measured by the latex agglutination method (Hyaluronic acid LT, Wako Pure Chemical Industries, Osaka, Japan).

Statistical analysis

Quantitative values are expressed as means ± SD. Between-group differences were analyzed by the chi-square test. Differences in quantitative values between two groups were analyzed by the Mann–Whitney *U* test. Correlation between liver tissue mRNA and serum levels of TIMP1 and MMP2 were evaluated with Spearman's test. Multivariate analysis was performed using logistic regression models. All *p* values were 2-tailed, and $p < 0.05$ was considered to indicate statistical significance.

Results

Background characteristics of study patients

Table 1 summarizes the characteristics of the study patients. Pathologic examination revealed that all patients had steatosis involving at least 10% of the hepatocytes, and NASH was diagnosed in 43 patients (67.2%). Among 43 patients diagnosed with NASH, 13 patients (30.2%) were diagnosed with stage 3 or 4 fibrosis according to the Brunt classification (Brunt et al. 1999). No significant differences were found between patients with and without NASH with respect to age, sex, body weight,

laboratory data, and degree of steatosis on pathologic evaluation (Supplemental Table 1).

Expression of TIMP1, TIMP2, MMP2, and MMP13 mRNA in liver tissue in patients with NAFLD

Figure 1 compares the gene expression levels of TIMP1, TIMP2, MMP2, and MMP13 based on the quantification of mRNA in the liver tissue of patients with and without NASH. The quantity of MMP2 mRNA was significantly higher in patients with NASH (2.69 ± 1.40, relative expression level) than those without (1.50 ± 0.57; $p < 0.0001$). No significant differences were found in the quantity of TIMP1, TIMP2, and MMP13 mRNA. There were no differences in the quantity of TIMP1, TIMP2, MMP2, and MMP13 mRNA according to the degree of steatosis (data not shown).

Table 1. Characteristics of study patients ($n = 64$).

Age (years)	51.0 ± 15.0
Sex (male/female)	36 (56.3)/28 (43.7)
Body weight (kg)	70.3 ± 11.5
Body mass index (kg/m^2)	27.1 ± 3.5
Alanine aminotransferase (IU/L)	88.5 ± 76.4
Aspartate aminotransferase (IU/L)	54.3 ± 32.5
Gamma-glutamyl transpeptidase (IU)	87.1 ± 64.6
Total bilirubin (mg/dL)	0.71 ± 0.54
Albumin (g/dL)	4.22 ± 0.51
Glucose (mg/dL)*	131.6 ± 61.3
Total cholesterol (mg/dL)*	197.8 ± 39.4
Triglyceride (mg/dL)*	162.1 ± 84.3
Hemoglobin A_1-c (%)	6.07 ± 1.55
Hemoglobin (g/dL)	14.7 ± 1.6
Platelet count (×10^3/μL)	238 ± 70
Ferritin (ng/mL)	231.0 ± 190.7
Steatosis (<30%/30–50%/50–70%/70%≤)**	17 (26.6)/18 (28.1)/19 (29.7)/10 (15.6)
Diagnosis (NASH/simple steatosis)	43 (67.2)/21 (32.8)
Fibrosis (grade 1/2/3/4)**,*	20 (46.5)/10 (23.3)/10 (23.3)/3 (6.9)

NASH, non-alcoholic steatohepatitis.
*Measured under fasting conditions.
**Based on pathologic examination.
*Only in patients with NASH by Brunt classification (Brunt 1999).

Serum levels of TIMP1, MMP2, and hyaluronic acid in patients with NAFLD

Figure 2 compares the levels of TIMP1, MMP2, and hyaluronic acid in serum samples obtained at the time of liver biopsy between patients with and without NASH. Serum levels of TIMP1 and MMP2 showed a significant correlation with liver tissue mRNA levels, respectively, although the correlation was not strong ($p = 0.0003$ and $\rho = 0.472$ for TIMP1, and $p < 0.0001$ and $\rho = 0.534$ for MMP2, Supplemental Figure 1). The serum levels of MMP2 and hyaluronic acid were significantly higher in patients with NASH than in patients without (MMP2, $p = 0.0198$, and hyaluronic acid, $p = 0.0042$). No significant differences were found in the serum levels of TIMP1 between patients with and without NASH.

Univariate and multivariate analyses were performed for factors associated with NASH (Table 2). In the univariate analysis, serum aspartate aminotransferase (AST), MMP2, and hyaluronic acid levels were associated with NASH. In multivariate analysis, serum AST, MMP2, and hyaluronic acid levels were independently associated with NASH.

Expression of MMP2 mRNA in liver tissue and serum levels of MMP2, and hyaluronic acid in NASH patients with mild fibrosis

When excluding patients with NASH and advanced fibrosis (Brunt's fibrosis stage [Brunt et al. 1999] 3 or 4), the quantity of hepatic MMP2 mRNA remained significantly higher in patients with NASH than those without ($p = 0.0010$, Figure 3). Serum levels of MMP2 were also significantly higher in patients with NASH than those without ($p = 0.0020$, Figure 3). No significant differences in serum levels of hyaluronic acid were observed ($p = 0.1296$, Figure 3). In both univariate and multivariate analyses, only serum AST and MMP2 levels were associated with NASH (Table 3).

The predictive value of serum levels of MMP2 and hyaluronic acid were analyzed with receiver-operating characteristic (ROC) analysis. In all patients, serum MMP2 and hyaluronic acid levels had comparable ability for predicting NASH among patients with NAFLD with

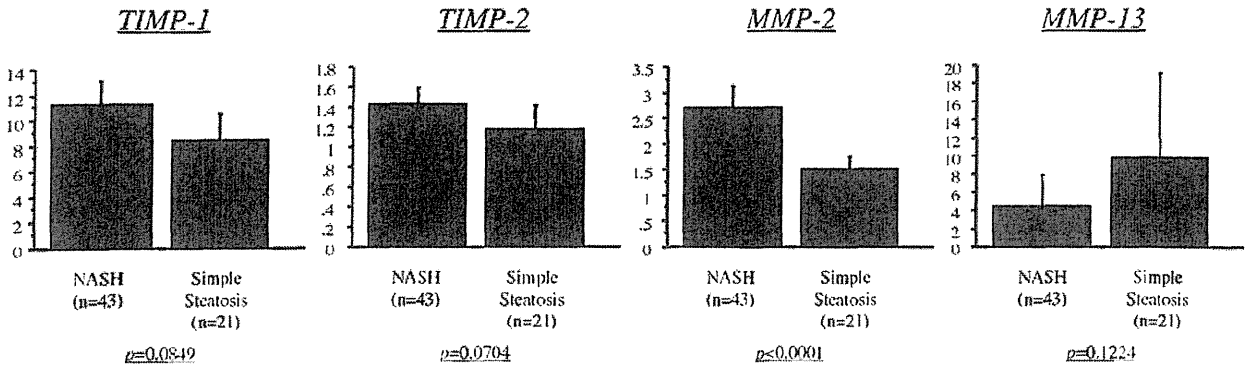

Figure 1. Relative mRNA expression levels of tissue inhibitor metalloproteinase-1 and -2 (TIMP1 and TIMP2), and matrix metalloproteinase 2 and 13 (MMP2 and MMP13) in the liver tissue of patients with nonalcoholic steatohepatitis (NASH) versus simple steatosis.

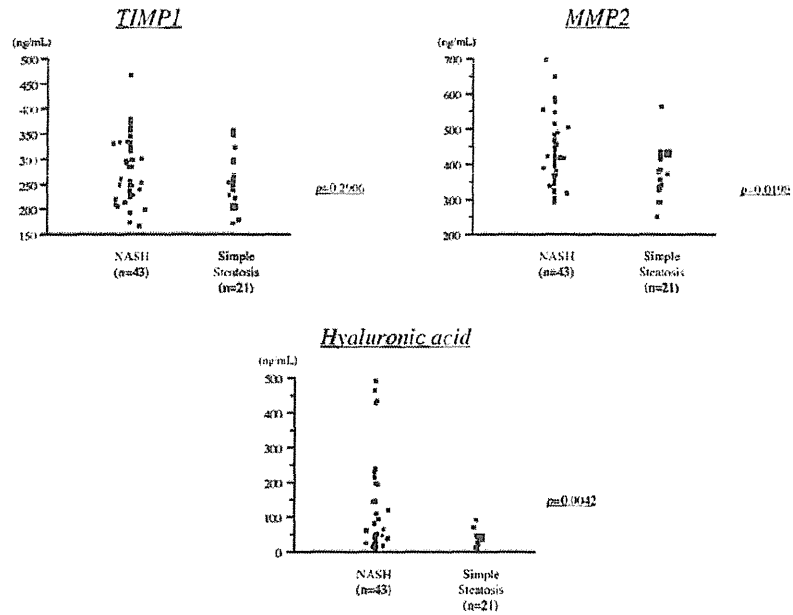

Figure 2. Serum levels of tissue inhibitor metalloproteinase-1 (TIMP1), matrix metalloproteinase-2 (MMP2), and hyaluronic acid in all patients with nonalcoholic steatohepatitis (NASH) versus simple steatosis. TIMP1, 277.4 ± 64.6 ng/mL with NASH vs. 256.5 ± 55.6 ng/mL with simple steatosis; $p = 0.2906$, MMP2, 427.8 ± 95.2 ng/mL with NASH vs. 353.6 ± 55.1 ng/mL with simple steatosis; $p = 0.0198$, and hyaluronic acid, 111.0 ± 131.5 ng/mL with NASH vs. 29.3 ± 21.2 ng/mL with simple steatosis; $p = 0.0042$.

Table 2. Univariate and multivariate analyses for distinguishing between patients with NASH and simple steatosis ($n = 64$).

	Univariate analysis (p value)	Multivariate analysis (p value)	Odds ratio (95% confidence interval)
Age (years)	0.4523		
Sex (male/female)	0.9199	–	
Body weight (kg)	0.4524	–	
Body mass index (kg/m²)	0.2999	–	
Alanine aminotransferase (IU/L)	0.2061	–	
Aspartate aminotransferase (IU/L)	0.0146	0.0258	938.371 (4.9097–922101.2)
Gamma-glutamyl transpeptidase (IU)	0.5724	–	
Total bilirubin (mg/dL)	0.1126	–	
Albumin (g/dL)	0.1959	–	
Glucose (mg/dL)*	0.1898	–	
Total cholesterol (mg/dL)*	0.2338	–	
Triglyceride (mg/dL)*	0.1696	–	
Hemoglobin A_1-c (%)	0.9370	–	
Hemoglobin (g/dL)	0.5974		
Platelet count (×10³/μL)	0.0613	–	
Ferritin (ng/mL)	0.5443	–	
TIMP1 (ng/mL)	0.2224	–	
MMP2 (ng/mL)	0.0058	0.0275	364.171 (3.9968–174225.9)
Hyaluronic acid (ng/mL)	0.0228	0.0351	23346.68 (32.5694–298598)
Steatosis (<30%/30–50%/50–70%/70%≤)**	0.8713	–	

NASH, non-alcoholic steatohepatitis; TIMP1, tissue inhibitor metalloproteinase-1; MMP2, matrix metalloproteinase-2.
*Measured during fasting conditions.
**Based on pathologic examination.

similar area under the ROC curves (AUROC) (MMP2, 0.73 and hyaluronic acid, 0.77, Supplemental Figure 2). When NASH patients with advanced fibrosis were excluded, the ability of serum hyaluronic acid levels to predict NASH decreased (AUROC, 0.63), whereas the predictive ability of serum MMP2 levels remained similar (AUROC, 0.74, Supplemental Figure 3).

Discussion

In the present study, we observed enhanced gene expression of MMP2 in liver tissue, along with elevated serum levels of MMP2, both of which showed the correlation, in patients with NASH compared to those with simple steatosis. MMP2 expression reportedly increases during the

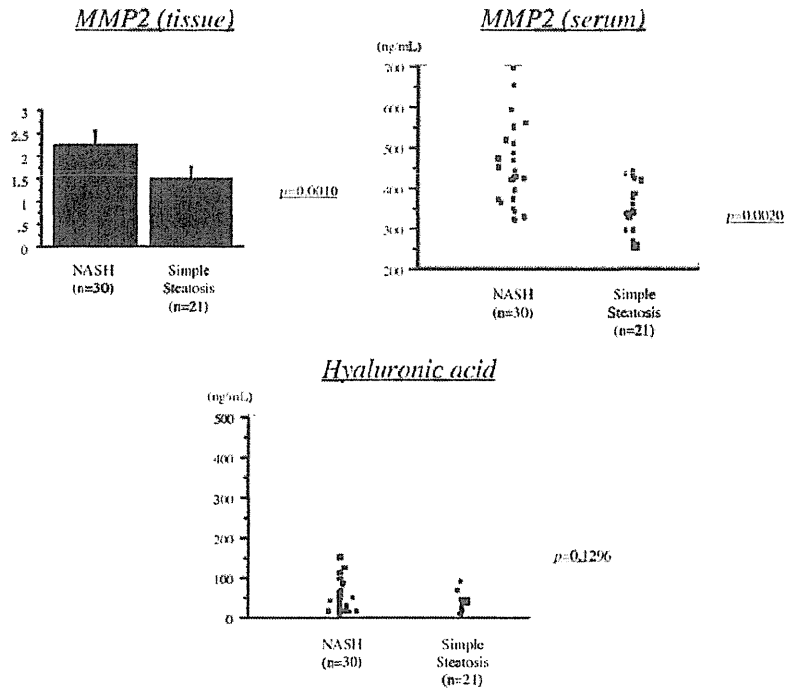

Figure 3. Quantity of matrix metalloproteinase-2 (MMP2) mRNA in liver tissue and serum levels of MMP2, and hyaluronic acid in nonalcoholic steatohepatitis (NASH) patients without advanced fibrosis versus patients with simple steatosis. MMP2 mRNA, 2.23 ± 0.84 with NASH vs. 1.50 ± 0.57 with simple steatosis; p = 0.0010, serum levels of MMP2, 441.8 ± 99.6 ng/mL with NASH vs. 353.6 ± 55.1 ng/mL with simple steatosis; p = 0.0020, and serum levels of hyaluronic acid, 143.1 ± 31.1 ng/mL with NASH vs. 130.3 ± 63.1 ng/mL with simple steatosis; p = 0.1296.

Table 3. Univariate and multivariate analyses for distinguishing patients with NASH and simple steatosis, excluding NASH patients with advanced fibrosis (n = 51).

	Univariate analysis (p value)	Multivariate analysis (p value)	Odds ratio (95% confidence interval)
Age (years)	0.7699	–	
Sex (male/female)	0.9730	–	
Body weight (kg)	0.3069	–	
Body mass index (kg/m^2)	0.2221	–	
Alanine aminotransferase (IU/L)	0.1769	–	
Aspartate aminotransferase (IU/L)	0.0309	0.0251	240.057 (3.5678–72252.5)
Gamma-glutamyl transpeptidase (IU)	0.7185	–	
Total bilirubin (mg/dL)	0.1344	–	
Albumin (g/dL)	0.3718	–	
Glucose (mg/dL)*	0.5584	–	
Total cholesterol (mg/dL)*	0.5173	–	
Triglyceride (mg/dL)*	0.1327	–	
Hemoglobin A_1-c (%)	0.7368	–	
Hemoglobin (g/dL)	0.8608	–	
Platelet count (×10^3/μL)	0.5635	–	
Ferritin (ng/mL)	0.8109	–	
TIMP1 (ng/mL)	0.2302	–	
MMP2 (ng/mL)	0.0047	0.0068	2759.72 (19.7697–2163013)
Hyaluronic acid (ng/mL)	0.1007	–	
Steatosis (<30%/30–50%/50–70%/70%≤)**	0.2877	–	

NASH, non-alcoholic steatohepatitis; TIMP1, tissue inhibitor metalloproteinase-1; MMP2, matrix metalloproteinase-2.
*Measured during fasting conditions.
**Based on pathologic examination.

progression of liver fibrosis (Ebata et al. 1997; Hemmann et al. 2007), and our results indicated that liver fibrosis is proceeding in patients with NASH.

NASH is usually diagnosed by histological evaluation of specimens obtained by liver biopsy and this is currently the only reliable and accepted method for the evaluation of liver fibrosis. However, liver biopsy is invasive and carries the risk of intraperitoneal bleeding. Therefore, noninvasive indicators of NASH in NAFLD patients would be important. Several biomarkers have been studied as indicators of NASH in patients with NAFLD (Malik et al. 2009; Miele et al. 2009). The serum level of hyaluronic acid is an important marker for the identification of patients with NASH. Because serum hyaluronic acid levels increase with the progression of liver fibrosis (Adams 2011) and the increase can simply reflect accumulated fibrosis in the liver as a result of the progression of NASH, it may not be an indicator of NASH but with mild fibrosis (i.e. early stage of NASH). Although serum hyaluronic acid levels were significantly higher in patients with NASH than those without NASH in the present study, we failed to find significant differences in serum hyaluronic acid levels between NASH patients with mild fibrosis and those without NASH. Whereas the AUROC for serum hyaluronic acid level in predicting NASH was more than 0.7 in patients including advanced fibrosis in the study by Malik et al. and in the present study, it decreased from 0.77 to 0.63 when focusing on patients with mild fibrosis in the present study. Serum hyaluronic acid levels, therefore, do not appear to be useful for distinguishing NASH patients with mild fibrosis from patients without NASH. In contrast, the AUROC for serum MMP2 levels in predicting NASH remains greater than 0.73 even in the subpopulation with mild fibrosis. In clinical practice, it will be important to identify NASH patients with mild fibrosis, before the progression of liver fibrosis caused by NASH, from NAFLD patients. Serum MMP2 levels may be useful for this purpose.

Conclusion

In patients with NASH, gene expression of MMP2, a protein associated with liver fibrosis, was enhanced in the liver tissue and serum levels of MMP2 were increased, indicating the initiation of liver fibrosis in this subpopulation. These results were also observed when NASH patients with advanced fibrosis were excluded. MMP2 may be a noninvasive indicator of early stage of NASH in patients with NAFLD.

Declaration of interest

The authors declare no conflicts of interest.

References

Adams LA. (2011). Biomarkers of liver fibrosis. *J Gastroenterol Hepatol* 26:802–809.

Angulo P. (2002). Nonalcoholic fatty liver disease. *N Engl J Med* 346:1221–1231.

Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. (1999). Nonalcoholic steatohepatitis: A proposal for grading and staging the histological lesions. *Am J Gastroenterol* 94:2467–2474.

Chitturi S, Farrell GC, Hashimoto E, Saibara T, Lau GK, Sollano JD; Asia-Pacific Working Party on NAFLD. (2007). Non-alcoholic fatty liver disease in the Asia-Pacific region: Definitions and overview of proposed guidelines. *J Gastroenterol Hepatol* 22:778–787.

Clark JM, Brancati FL, Diehl AM. (2003). The prevalence and etiology of elevated aminotransferase levels in the United States. *Am J Gastroenterol* 98:960–967.

Ebata M, Fukuda Y, Nakano I, Katano Y, Fujimoto N, Hayakawa T. (1997). Serum levels of tissue inhibitor of metalloproteinases-2 and of precursor form of matrix metalloproteinase-2 in patients with liver disease. *Liver* 17:293–299.

Fassio E, Alvarez E, Domínguez N, Landeira G, Longo C. (2004). Natural history of nonalcoholic steatohepatitis: A longitudinal study of repeat liver biopsies. *Hepatology* 40:820–826.

Hamaguchi M, Kojima T, Itoh Y, Harano Y, Fujii K, Nakajima T, Kato T, Takeda N, Okuda J, Ida K, Kawahito Y, Yoshikawa T, Okanoue T. (2007). The severity of ultrasonographic findings in nonalcoholic fatty liver disease reflects the metabolic syndrome and visceral fat accumulation. *Am J Gastroenterol* 102:2708–2715.

Hashimoto E, Yatsuji S, Kaneda H, Yoshioka Y, Taniai M, Tokushige K, Shiratori K. (2005). The characteristics and natural history of Japanese patients with nonalcoholic fatty liver disease. *Hepatol Res* 33:72–76.

Hemmann S, Graf J, Roderfeld M, Roeb E. (2007). Expression of MMPs and TIMPs in liver fibrosis - a systematic review with special emphasis on anti-fibrotic strategies. *J Hepatol* 46:955–975.

Kojima S, Watanabe N, Numata M, Ogawa T, Matsuzaki S. (2003). Increase in the prevalence of fatty liver in Japan over the past 12 years: Analysis of clinical background. *J Gastroenterol* 38:954–961.

Malik R, Chang M, Bhaskar K, Nasser I, Curry M, Schuppan D, Byrnes V, Afdhal N. (2009). The clinical utility of biomarkers and the nonalcoholic steatohepatitis CRN liver biopsy scoring system in patients with nonalcoholic fatty liver disease. *J Gastroenterol Hepatol* 24:564–568.

Matteoni CA, Younossi ZM, Gramlich T, Boparai N, Liu YC, McCullough AJ. (1999). Nonalcoholic fatty liver disease: A spectrum of clinical and pathological severity. *Gastroenterology* 116:1413–1419.

Miele L, Forgione A, La Torre G, Vero V, Cefalo C, Racco S, Vellone VG, Vecchio FM, Gasbarrini G, Rapaccini GL, Neuman MG, Grieco A. (2009). Serum levels of hyaluronic acid and tissue metalloproteinase inhibitor-1 combined with age predict the presence of nonalcoholic steatohepatitis in a pilot cohort of subjects with nonalcoholic fatty liver disease. *Transl Res* 154:194–201.

Musso G, Gambino R, Cassader M. (2010). Non-alcoholic fatty liver disease from pathogenesis to management: An update. *Obes Rev* 11:430–445.

Torres DM, Harrison SA. (2008). Diagnosis and therapy of nonalcoholic steatohepatitis. *Gastroenterology* 134:1682–1698.

54：608　　消化器内科，54(5)：608-613，2012

特集II　B型肝炎の抗ウイルス療法の進歩と耐性

B型肝炎に対する核酸アナログ投与例の長期予後*

多田俊史** 熊田　卓**
桐山勢生** 谷川　誠**
豊田秀徳** 久永康宏**
金森　明** 新家卓郎**
安東直人** 坂井圭介**
安田　諭** 木村　純**
安藤祐資** 山本健太**

Key Words : hepatitis B, nucleos(t)ide analogue, carcinogenesis, hepatitis B virus core-related antigen, hepatocellular carcinoma

はじめに

B型肝炎は全世界において3億5000万人の持続感染者の存在が推測されており，年間60～100万人がB型肝炎に起因する慢性肝炎，肝硬変，肝癌で死亡していると推測されている[1]．近年，B型肝炎に対する治療薬である核酸アナログ製剤が登場し，それ以前に使用されることが多かったインターフェロンと比較して副作用が少なく，抗ウイルス効果が高いため，臨床の現場で広く使用されるようになり，B型肝炎の治療環境は大きく変化してきている．加えて免疫抑制剤・化学療法により発症するB型肝炎(HBVの再活性化)が注目されるようになり[2]，核酸アナログ投与例はさらに増加してきている．

今回われわれは，当院で経験したB型肝炎患者における核酸アナログ投与と肝発癌に関する検討を行ったので若干の考察を含め報告する．

対象と方法

1998～2008年の10年間に当院で経験したB型肝炎患者1,973例中，①HBs抗原が6か月以上陽性，②経過観察開始から3年以上経過，③alanine aminotransferase(ALT)を年2回以上測定，④発癌例では経過観察開始後1年以降で発癌，⑤核酸アナログ服用例では1年以上服用，のすべてを満たしたのは785例であった．これら785例中，核酸アナログ投与例は148例で，非投与例は637例であった．さらにpropensity score matching法を用いて経過観察開始時の背景因子(年齢，性別，ALT，血小板，HBV-DNA量，HBe抗原，Child-Pugh分類)をマッチさせ，核酸アナログ投与群および非投与群をそれぞれ117例ずつ選択し，計234例を今回の対象とした(図1)．

核酸アナログ投与群および非投与群の患者背景を表1に示す．背景因子として，治療開始時もしくは経過観察開始時の年齢，性別，ジェノタイプ(A/B/C)，HBV-DNA量，HBe抗原，プレコアと基本コアプロモーター(BCP)の野生型/変異型，血小板，ALTに有意差は認められなかった．

核酸アナログ製剤の投与状況は，ラミブジンが18例，ラミブジン＋アデフォビルが28例，エンテカビルが71例(エンテカビル開始が30例，ラミブジンからエンテカビルに変更が41例)であった．また，観察期間中央値は7.1年(1.5～18.3)であった．

* Long-term outcome of hepatitis B under treatment with nucleos(t)ide analogues.

** Toshifumi TADA, M.D., Takashi KUMADA, M.D., Seiki KIRIYAMA, M.D., Makoto TANIKAWA, M.D., Hidenori TOYODA, M.D., Yasuhiro HISANAGA, M.D., Akira KANAMORI, M.D., Takuro NIINOMI, M.D., Naoto ANDO, M.D., Keisuke SAKAI, M.D., Satoshi YASUDA, M.D., Jun KIMURA, M.D., Yusuke ANDO, M.D. & Kenta YAMAMOTO, M.D.: 大垣市民病院消化器内科[〒503-8502 岐阜県大垣市南頬町4-86]；Department of Gastroenterology, Ogaki Municipal Hospital, Ogaki, Gifu 503-8502, JAPAN

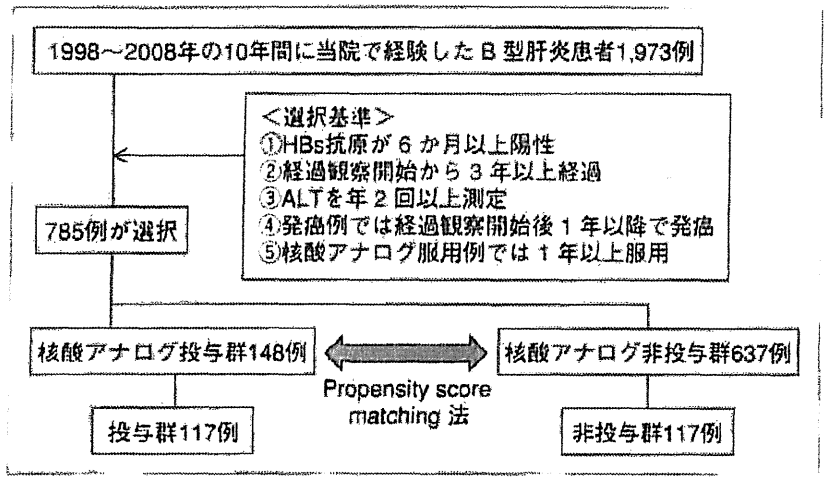

図1 対象患者の選択

表1 患者背景

	投与群(n=117)	非投与群(n=117)	P値
年齢(歳)	52(27～77)	52(21～77)	0.922
性別(女性/男性)	44/73	45/72	0.893
ジェノタイプ(A/B/C)	1/4/109	4/7/85	0.123
HBV-DNA(log copies/ml)	6.7(2.6～9.6)	6.5(2.1～9.6)	0.084
HBeAg(+/−)	57/60	58/59	0.896
プレコア(野生型/変異型)	22/87	16/75	0.640
BCP(野生型/変異型)	22/88	17/70	0.936
血小板(×10⁴/m³)	14.3(3.2～26.2)	14.6(3.7～39.6)	0.634
ALT(IU/ml)	68(7～1,088)	55(9～3,410)	0.098

検討内容は血小板，ALT，gamma glutamyl transpeptidase(γ-GTP)，total bilirubin(T. bil)，alkaline phosphatase(ALP)，albumin(Alb)，alpha-fetoprotein(AFP)，HBV-DNA量の各項目に対して，治療開始時もしくは経過観察開始時および経過観察開始後の時間軸を考慮に入れた積分平均値[7]を算出し，核酸アナログ投与群と非投与群の比較を行った．なお，発癌例の血液データは発癌1年前までの値を使用した．さらに，発癌率および多変量解析を用い発癌に関与する因子につき検討を行った．また，HBコア関連抗原(HBcrAg)の測定が可能であった一部の症例では，HBcrAgと発癌との関連についても検討を行った．

統計ソフトはSPSS(ver 18)を用いて行い，2群間の比較はMann-WhitneyのU検定，発癌率の検定はKaplan-Meier法，多変量解析にはCox比例ハザードモデル(変数増加法)をそれぞれ使用し，$P<0.05$を有意差ありと判定した．

結　果

経過観察開始後の血液・生化学データ(積分平均値)を表2に示す．核酸アナログ投与群は非投与群と比較して，血小板が高値($P=0.006$)，ALTが低値($P<0.001$)，γ-GTPが低値($P=0.043$)，ALPが低値(0.013)，Albが高値($P<0.001$)，AFPが低値($P<0.001$)となり肝機能は明らかに改善が認められた．HBV-DNA量(積分平均値)に関しても同様に核酸アナログ投与群は2.5log copies/ml(1.2～8.9)，非投与群は4.6log copies/ml(2.1～9.3)で，投与群において有意な低下が認められた($P<0.001$)(図2)．

続いて，治療開始時もしくは経過観察開始時の年齢(40歳以下/超)，性別，核酸アナログ投与の有無，プレコア，BCP，HBV-DNA量(5.0log copies/ml以下/超)，HBcrAg(3.0log U/ml以下/超)，血小板(15×10^4/m³以下/超)，ALT(40IU/l以下/超)，γ-GTP(56IU/l以下/超)，ALP(338IU/l以下/