Table III. Comparison of characteristics between low insulin group and high insulin group.

Variable	Number or mean (SD)		P
	Low insulin group (n=70)	High insulin group (n=70)	
Onset age, y.o.	64.7 (9.5)	65.5 (9.5)	0.620
Gender			0.999
Male	55	55	
Female	15	15	
BMI, kg/m^2	22.5 (3.0)	23.7 (3.0)	0.019
Alcohol intake			0.687
<80 g/day	53	55	
≥80 g/day	17	15	
Etiology			0.818
HBsAg(+)	16	13	
HCVAb(+)	48	51	
Non-B, non-C	6	6	
Underlying liver diseases and Child-Pugh grade			0.539
CH	17	13	
LC grade A	37	35	
LC grade B	14	17	
LC grade C	2	5	
Total bilirubin, ng/ml	1.5 (2.9)	1.3 (1.3)	0.634
Ferritin, ng/ml	305.2 (351.7)	303.2 (341.2)	0.974
Serum iron, μg/ml	155 (77)	148 (74)	0.571
Fasting insulin, μIU/ml	4.3 (2.2)	15.8 (10.6)	<0.001
Fasting blood glucose, mg/dl	94.0 (16.6) (n=31)	111.4 (70.5) (n=31)	0.185
HOMA-R, %	0.9 (0.6) (n=13)	4.8 (2.4) (n=15)	<0.001
Total adiponectin, μg/ml	7.9 (4.3)	8.3 (5.3)	0.611
HMW, μg/ml	3.7 (2.7)	4.1 (3.3)	0.473
MMW, μg/ml	1.9 (1.2)	1.8 (1.1)	0.570
LMW, μg/ml	2.3 (1.1)	2.5 (1.4)	0.600

calculated in 28 patients, and the level of HOMA-R in the high insulin group was significantly higher than that in the low insulin group. Table IV shows the comparison of characteristics of HCC between the two groups. Patients with more than three HCC lesions and diffuse HCC were more prevalent in the low insulin group than in the high insulin group. The other characteristics of HCC did not differ substantially between the two groups.

Fig. 2A indicates the cumulative survival rates of all stage HCC patients between the low insulin group (70 of 140) and the high insulin group (70 of 140). There was no significant difference between the two groups (P=0.235). Next, to evaluate the relationship of the fasting insulin level with the prognosis of early stage HCC patients, we analyzed the cumulative survival rates in HCC patients with TNM stage I and II disease (n=92). As shown in Fig. 2B, the high insulin group (49 of 92) exhibited a poor prognosis with a significant difference in comparison to the low insulin group (43 of 92) (P=0.018).

Association of fasting total adiponectin level with prognosis of HCC. Similarly, we evaluated the association of the total adiponectin level with the prognosis of HCC. One hundred and forty patients were divided into 2 groups in terms of the 50th percentile of the value of total adiponectin (6.95 μIU/ml). The mean level of total adiponectin in the low adiponectin group (<6.95 μIU/ml, n=70) was 4.5 μg/ml. That in the high adiponectin group (≥6.95 μIU/ml, n=70) was 11.8 μg/ml. We estimated the cumulative survival rates of all stages of HCC and early stage HCC between the low adiponectin group and the high adiponectin group. Fig. 3A and B show each result. No significant differences were found in all stages of HCC, or in the early stage of HCC (all stage HCC: low group vs. high group; P=0.886, early stage HCC: low group vs. high group; P=0.804).

Univariate and multivariate analyses of the factors associated with HCC prognosis. Univariate and multivariate analyses

Table IV. Comparison of HCC characteristics between low insulin group and high insulin group.

Variable	Number or mean (SD)		P
	Low insulin group (n=70)	High insulin group (n=70)	
Tumor size, cm	3.6 (3.2)	3.2 (2.5)	0.479
Number of tumor lesion			0.032
1	36	39	
2	10	19	
3 - and diffuse	24	12	
TNM stage			0.300
I	22	17	
II	21	32	
III	21	16	
IV	6	5	
AFP, ng/ml	5136.8 (30566.9)	10487.6 (83045.4)	0.614
Therapy			0.492
Surgical resection	5	2	
PEIT and/or RFA	27	26	
TACE or TAI	35	38	
Others	3	4	

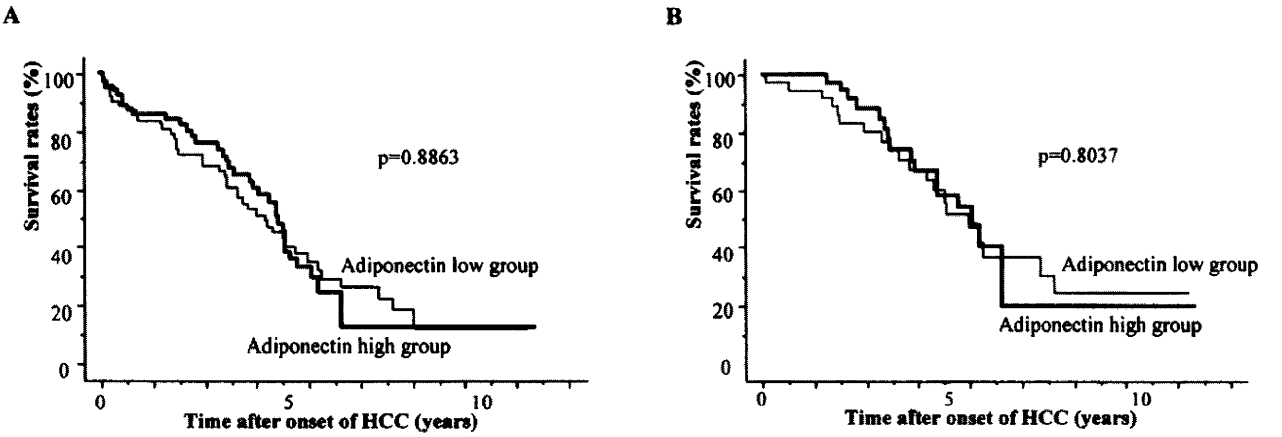


Figure 3. Kaplan-Meier curves for survival between low adiponectin group (thin line) and high adiponectin group (heavy line) in all stages of HCC patients (n=140) (A) and in TNM stage I + II HCC patients (n=92) (B).

using the Cox proportional hazards model in 140 patients diagnosed with HCC were performed to identify the relevant independent prognostic factors in all stages of HCC. In a univariate analysis, the following three factors significantly influenced the prognosis: alcohol intake (excessive drinker, RR 2.033, 95% CI 1.206-3.425, P=0.008), Child-Pugh grade (grade C, RR 9.906, 95% CI 3.547-27.666, P<0.001), and therapy for HCC (TACE or TAI, RR 1.856, 95% CI 1.143-3.015, P=0.012). However, a multivariate analysis revealed that only two factors influenced the HCC prognosis significantly: Child-Pugh grade (grade C, RR 9.807, 95% CI 2.710-30.471, P<0.001) and therapy for HCC (TACE or TAI, RR 1.803, 95% CI 1.104-2.943, P=0.018).

Next, univariate and multivariate analyses in 92 patients diagnosed with HCC, all TNM stage I or II, were performed to identify the independent prognostic factors of early stage HCC. In the univariate analysis, the following three factors significantly influenced prognosis: alcohol intake (excessive drinker, RR 2.488, 95% CI 1.160-5.319, P=0.019), Child-Pugh grade (grade B, RR 4.582, 95% CI 1.370-15.323, P=0.014, grade C, RR 41.104, 95% CI 6.403-263.831, P<0.001), and the value of insulin (>7.73 μIU/ml, RR 2.196, 95% CI 1.126-4.292, P=0.021) (Table V).

Similarly, when we performed multivariate analysis, only two factors, the Child-Pugh grade and the level of fasting insulin influenced the prognosis of early stage HCC with a

Table V. Univariate analyses of prognosis factors for HCC of TNM stage I and II.

Variable	Relative risk (95% CI)	P
Onset age, >60 y.o.	1.248 (0.536-2.907)	0.606
Gender, male	1.637 (0.715-3.745)	0.244
BMI, >25.0 kg/m^2	1.488 (0.746-2.967)	0.260
Alcohol intake, ≥80g/day	2.488 (1.160-5.319)	0.019
Background		0.647
non-B, non-C	-	-
HBsAg(+)	1.111 (0.230-5.369)	0.896
HCVAb(+)	1.566 (0.366-6.700)	0.545
Underlying liver diseases and Child-Pugh grade		<0.001
CH	-	-
LC Child-Pugh grade A	2.531 (0.866-7.395)	0.090
LC Child-Pugh grade B	4.582 (1.370-15.323)	0.014
LC Child-Pugh grade C	41.104 (6.403-263.831)	<0.001
Serum ferritin, <185 ng/ml	1.193 (0.621-2.295)	0.596
Serum iron, <141 μg/ml	1.222 (0.641-2.331)	0.542
Fasting insulin, >7.73 μIU/ml	2.196 (1.126-4.292)	0.021
Fasting blood glucose, >110 mg/dl	0.949 (0.118-7.634)	0.961
HOMA-R, >2.0%	4.762 (0.475-47.619)	0.184
Total adiponectin, >6.95 μg/ml	0.921 (0.479-1.767)	0.804
HMW, 3.0 μg/ml	0.799 (0.418-1.529)	0.498
MMW, >1.6 μg/ml	1.171 (0.613-2.232)	0.633
LMW, >2.1 μg/ml	1.038 (0.544-1.984)	0.908
Therapy, TACE or TAI	1.429 (0.743-2.748)	0.285

Table VI. Multivariate analyses of prognosis factors for HCC of TNM stage I and II.

Variable	Relative risk (95% CI)	P
Alcohol intake, ≥80 g/day	2.217 (0.933-5.263)	0.071
Underlying liver diseases and Child-Pugh grade		0.022
CH	-	-
LC Child-Pugh grade A	2.884 (0.975-8.531)	0.056
LC Child-Pugh grade B	3.771 (1.099-12.529)	0.035
LC Child-Pugh grade C	19.039 (2.782-130.298)	0.003
Fasting insulin, >7.73 μIU/ml	2.033 (1.019-4.049)	0.044

significant difference: Child-Pugh grade (grade B, RR 3.771, 95% CI 1.099-12.529, P=0.035, grade C, RR 19.039, 95% CI 2.782-130.298, P=0.003), and the level of fasting insulin (>7.73 μIU/ml, RR 2.033, 95% CI 1.019-4.049, P=0.044) (Table VI).

Association of fasting insulin and total adiponectin level with recurrence-free survival. To evaluate the association of fasting insulin level with the recurrence-free survival time, 59 patients who underwent curative therapy, defined as a condition characterized by the no findings of recurrence over six months after the initial therapy, were extracted from 140 patients and subjected to analysis. Of 59 patients, the mean level of insulin in the low insulin group (<7.73 μIU/ml, n=32) or that in the high insulin group (>7.73 μIU/ml, n=27) was 3.8 μIU/ml or 14.4 μIU/ml, respectively. Fig. 4A indicates the cumulative recurrence-free survival rates of 59 patients who underwent curative therapy. The high insulin group exhibited a lower recurrence-free survival with a significant difference in comparison to the low insulin group (P=0.017).

Similarly, we evaluated the association of the total adiponectin level with the recurrence-free survival time. The mean level of total adiponectin in the low adiponectin group (<6.95 μIU/ml, n=28) or that in the high adiponectin group (≥6.95 μIU/ml, n=31) was 4.5 μIU/ml or 11.7 μIU/ml, respectively. We compared the cumulative recurrence-free survival rates of HCC between the low adiponectin group and the high adiponectin group, but no significant difference was found (Fig. 4B).

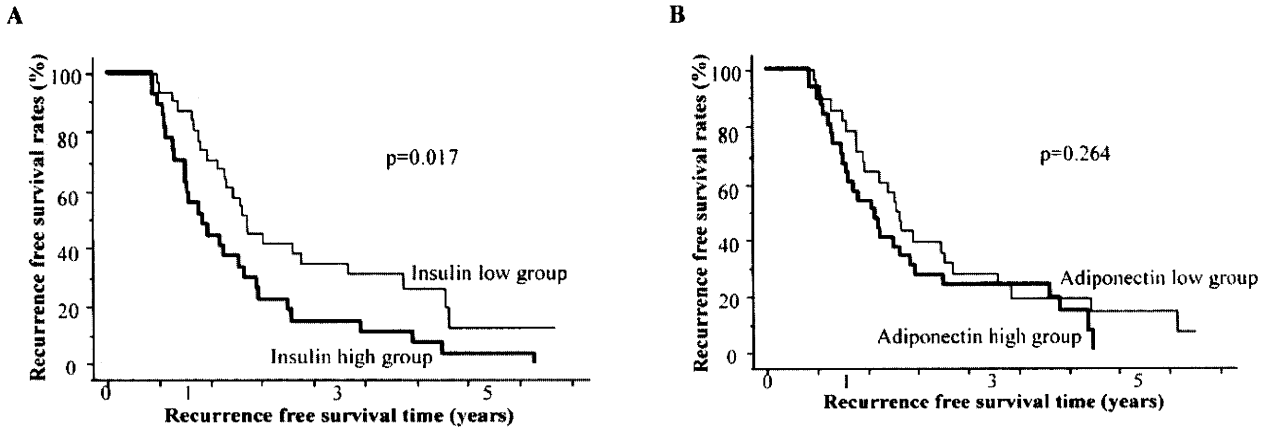


Figure 4. Kaplan-Meier curves for recurrence-free survival in HCC patients who underwent curative therapy (n=59) between low insulin group (thin line) and high insulin group (heavy line) (A) and between low adiponectin group (thin line) and high adiponectin group (heavy line) (B).

Table VII. Univariate analyses of the factor that contribute to recurrence-free survival.

Variable	Relative risk (95% CI)	P
Onset age, >60 y.o.	1.905 (0.951-3.871)	0.069
Gender, male	1.984 (0.355-1.243)	0.201
BMI, >25.0 kg/m^2	2.268 (0.805-4.367)	0.014
Alcohol intake, ≥80 g/day	1.289 (0.653-2.538)	0.465
Background		0.671
Non-B, non-C	-	-
HBsAg(+)	1.302 (0.289-5.855)	0.731
HCVAb(+)	1.566 (0.390-6.773)	0.505
Underlying liver diseases and Child-Pugh grade		0.093
CH	-	-
LC Child-Pugh grade A	2.300 (1.095-4.831)	0.028
LC Child-Pugh grade B	2.883 (1.086-7.650)	0.034
LC Child-Pugh grade C	3.655 (0.774-17.263)	0.102
Serum ferritin, <185 ng/ml	1.157 (0.663-2.019)	0.607
Serum iron, <141 μg/ml	1.379 (0.772-2.464)	0.278
Fasting insulin, >7.73 μIU/ml	1.946 (1.117-3.378)	0.019
Fasting blood glucose, >110 mg/dl	4.975 (0.903-27.778)	0.065
HOMA-R, >2.0%	4.255 (0.816-22.222)	0.086
Total adiponectin, >6.95 μg/ml	1.376 (0.784-2.410)	0.266
HMW, >3.0 μg/ml	1.076 (0.611-1.893)	0.799
MMW, >1.6 μg/ml	1.258 (0.711-2.222)	0.430
LMW, >2.1 μg/ml	1.012 (0.572-1.792)	0.967
Therapy, TACE or TAI	1.165 (0.646-2.101)	0.610

Univariate and multivariate analyses of the factors associated with recurrence-free survival. To clarify the factors that contribute to recurrence-free survival except for tumoral factors, univariate and multivariate analyses were performed using the Cox proportional hazards model in 59 patients who underwent curative therapy. In a univariate analysis, only two

Table VIII. Multivariate analyses of the factors that contribute to recurrence-free survival.

Variable	Relative risk (95% CI)	P
BMI, >25.0 kg/m^2	1.992 (1.026-3.861)	0.042
Fasting insulin, >7.73 μIU/ml	1.767 (1.004-3.117)	0.049

factors significantly influenced the recurrence-free survival: BMI (>25.0 kg/m^2, RR 2.268, 95% CI 0.805-4.367, P=0.014) and the value of insulin (>7.73 μIU/ml, RR 1.946, 95% CI 1.117-3.378, P=0.019) (Table VII). Multivariate analysis showed that both factors influenced the recurrence-free survival with a significant difference: BMI (>25.0 kg/m^2, RR 1.992, 95% CI 1.026-3.861, P=0.042) and the value of insulin (>7.73 μIU/ml, RR 1.767, 95% CI 1.004-3.117, P=0.049) (Table VIII).

Discussion

Several prior studies have reported that the coexistence of DM influences the prognosis of HCC patients (10,11,28,29). However, the mechanism responsible for this finding remains unclear. Since the glucose tolerance of an individual is defined by the potential insulin secretion from ß-cells and by the insulin sensitivity of target tissues including the liver, serum levels of fasting and postprandial insulin could differ in each HCC patient. In addition, advanced liver fibrosis is directly linked to an increase in the insulin resistance in HCV-infected patients (13,30).

In the present study, we therefore focused on the serum level of insulin rather than on the glucose tolerance in the HCC patients. Our study indicates that a high value of fasting insulin heralds not only a poor prognosis in the early stage of HCC but also a high recurrence rate in the curative HCC. There are a few studies on the prognostic value of hyperinsulinemia on patients with HCC. Saito *et al* have demonstrated that the area under the plasma insulin curve for the oral glucose tolerance test can serve as a significant prognostic tool, and can assist in forecasting the doubling time of HCC (16), and that continuous infusion of octreotide in five patients inhibited insulin secretion resulting in a decrease in the HCC growth rate.

Komura *et al* reported that insulin therapy for coexisting DM is an independent risk factor for HCC recurrence after a curative resection (10). Taken together, it is possible that hyperinsulinemia promotes the progression and development of HCC. This is consistent with the results from the following *in vitro* studies, that insulin has the potential to accelerate the growth of hepatoma cells and inhibits apoptosis through the upregulation of Bcl-xl (14), and that insulin stimulates the motility and invasiveness of hepatoma cells (31). In addition, there have been several clinical studies supporting the association between hyperinsulinemia and the advancement of cancers. A high level of fasting insulin is associated with distant recurrence and death in early stage breast cancer (32). High insulin levels are associated with a poorer prognosis in prostate cancer and endometrial cancer, and malignant degeneration of adenomatous polyps (33-36). These findings suggest that, in addition to an effect on glucose metabolism, insulin functions to promote the proliferation and metastasis of various types of cancer cells.

Hyperinsulinemia is inextricably linked to insulin resistance of the peripheral tissues including the liver. In our study, HOMA-R, a good indicator of insulin resistance, was not associated with a poor prognosis in early stage HCC (univariate analysis, P=0.184) and a recurrence-free survival in curative HCC (univariate analysis, P=0.086) although HOMA-R was significantly higher in the high insulin group than in the low insulin group (Table III). It is probably due to the small number of cases used to determine the HOMA-R (28 of 140 subjects or 11 of 59 subjects).

Since adiponectin has a potent insulin-sensitizing effect, we determined its value in HCC patients. In contrast to fasting serum insulin, the mean value of total adiponectin apparently increased with the decline of liver function. The HCC stage did not affect the values of total adiponectin. A similar observation has been reported by Tacke *et al* (23), in which they suggest that the elevation of adiponectin in chronic liver disease is due to the decrease of clearance from the serum, and possibly decreased biliary excretion of adiponectin, and that portal hypertension and the development of HCC do not affect the values of adiponectin. In addition to total adiponectin, we measured the levels of HMW, MMW, and LMW adiponectins. These adiponectins increased in direct relation to the decline in the liver function (data not shown), thus suggesting that higher molecular weight adiponectin is also metabolized by the liver. It is surprising that the values of total, HMW, MMW, and LMW adiponectins showed no significant differences between the high insulin group and the low group (Table III). However, Tacke *et al* have already reported a similar observation that the elevated adiponectin in LC patients is not directly involved in insulin sensitivity. Recently, adiponectin is known to possess antitumoral activity. The circulating adiponectin level is inversely associated with an increased risk of breast cancer, endometrial, prostate, gastric, and colorectal cancer (37-41). Furthermore, Miyazaki *et al* reported that adiponectin shows an antitumor effect against HepG2 hepatoma cells through JNK activation and suppression of STAT3 function (42). However, our study showed that total adiponectin has no impact on the prognosis of any stage of HCC. It is unclear why there is a discrepancy between these literature findings and our own. We are now speculating that certain cirrhotic environments such as advanced liver fibrosis, decreased liver function and portal systemic shunting may diminish the anti-tumoral activity of adiponectin against HCC. Further studies are thus needed to clarify this.

Although the present study is retrospective and involves a limited number of participants, this is a first study indicating that fasting hyperinsulinemia is a risk factor associated with a poor prognosis in the early stage of HCC and a high recurrence rate in the curative HCC. We have to validate our findings with a prospective study and also clarify the mechanism by which insulin impacts the clinical course of HCC. However, our study suggests that treatment modalities which lower the level of fasting insulin could improve the prognosis of the early stage of HCC and reduce the recurrence of HCC.

References

1. Bosch FX, Ribes J, Diaz M and Cleries R: Primary liver cancer: worldwide incidence and trends. Gastroenterology 127: S5-S16, 2004.
2. El-Serag HB and Mason AC: Risk factors for the rising rates of primary liver cancer in the United States. Arch Intern Med 160: 3227-3230, 2000.
3. Yu MC and Yuan JM: Environmental factors and risk for hepatocellular carcinoma. Gastroenterology 127: S72-S78, 2004.
4. Marchesini G, Brizi M, Morselli-Labate AM, *et al*: Association of nonalcoholic fatty liver disease with insulin resistance. Am J Med 107: 450-455, 1999.
5. Cotrim HP, Parana R, Braga E and Lyra L: Nonalcoholic steatohepatitis and hepatocellular carcinoma: natural history? Am J Gastroenterol 95: 3018-3019, 2000.
6. Shimada M, Hashimoto E, Taniai M, *et al*: Hepatocellular carcinoma in patients with non-alcoholic steatohepatitis. J Hepatol 37: 154-160, 2002.
7. Lagiou P, Kuper H, Stuver SO, Tzonou A, Trichopoulos D and Adami HO: Role of diabetes mellitus in the etiology of hepatocellular carcinoma. J Natl Cancer Inst 92: 1096-1099, 2000.
8. El-Serag HB, Richardson PA and Everhart JE: The role of diabetes in hepatocellular carcinoma: a case-control study among United States Veterans. Am J Gastroenterol 96: 2462-2467, 2001.
9. Davila JA, Morgan RO, Shaib Y, McGlynn KA and El-Serag HB: Diabetes increases the risk of hepatocellular carcinoma in the United States: a population based case control study. Gut 54: 533-539, 2005.
10. Komura T, Mizukoshi E, Kita Y, *et al*: Impact of diabetes on recurrence of hepatocellular carcinoma after surgical treatment in patients with viral hepatitis. Am J Gastroenterol 102: 1939-1946, 2007.
11. Huo TI, Wu JC, Lui WY, *et al*: Differential mechanism and prognostic impact of diabetes mellitus on patients with hepatocellular carcinoma undergoing surgical and nonsurgical treatment. Am J Gastroenterol 99: 1479-1487, 2004.
12. Perseghin G, Mazzaferro V, Sereni LP, *et al*: Contribution of reduced insulin sensitivity and secretion to the pathogenesis of hepatogenous diabetes: effect of liver transplantation. Hepatology 31: 694-703, 2000.
13. Taura N, Ichikawa T, Hamasaki K, *et al*: Association between liver fibrosis and insulin sensitivity in chronic hepatitis C patients. Am J Gastroenterol 101: 2752-2759, 2006.
14. Wanke I, Schwarz M and Buchmann A: Insulin and dexamethasone inhibit TGF-beta-induced apoptosis of hepatoma cells upstream of the caspase activation cascade. Toxicology 204: 141-154, 2004.
15. Tran TT, Medline A and Bruce WR: Insulin promotion of colon tumors in rats. Cancer Epidemiol Biomarkers Prev 5: 1013-1015, 1996.
16. Saito K, Inoue S, Saito T, *et al*: Augmentation effect of postprandial hyperinsulinaemia on growth of human hepatocellular carcinoma. Gut 51: 100-104, 2002.
17. Tschritter O, Fritsche A, Thamer C, *et al*: Plasma adiponectin concentrations predict insulin sensitivity of both glucose and lipid metabolism. Diabetes 52: 239-243, 2003.
18. Matsuzawa Y, Funahashi T, Kihara S and Shimomura I: Adiponectin and metabolic syndrome. Arterioscler Thromb Vasc Biol 24: 29-33, 2004.
19. Diez JJ and Iglesias P: The role of the novel adipocyte-derived hormone adiponectin in human disease. Eur J Endocrinol 148: 293-300, 2003.
20. Havel PJ: Update on adipocyte hormones: regulation of energy balance and carbohydrate/lipid metabolism. Diabetes 53 (Suppl 1): S143-S151, 2004.
21. Xu A, Wang Y, Keshaw H, Xu LY, Lam KS and Cooper GJ: The fat-derived hormone adiponectin alleviates alcoholic and nonalcoholic fatty liver diseases in mice. J Clin Invest 112: 91-100, 2003.
22. Tietge UJ, Boker KH, Manns MP and Bahr MJ: Elevated circulating adiponectin levels in liver cirrhosis are associated with reduced liver function and altered hepatic hemodynamics. Am J Physiol Endocrinol Metab 287: E82-E89, 2004.
23. Tacke F, Wustefeld T, Horn R, *et al*: High adiponectin in chronic liver disease and cholestasis suggests biliary route of adiponectin excretion in vivo. J Hepatol 42: 666-673, 2005.
24. Kaser S, Moschen A, Kaser A, *et al*: Circulating adiponectin reflects severity of liver disease but not insulin sensitivity in liver cirrhosis. J Intern Med 258: 274-280, 2005.
25. Hui CK, Zhang HY, Lee NP, *et al*: Serum adiponectin is increased in advancing liver fibrosis and declines with reduction in fibrosis in chronic hepatitis B. J Hepatol 47: 191-202, 2007.
26. Pugh RN, Murray-Lyon IM, Dawson JL, Pietroni MC and Williams R: Transection of the oesophagus for bleeding oesophageal varices. Br J Surg 60: 646-649, 1973.
27. Cancer Study Group of Japan: Clinical findings. In: The General Rules for the Clinical and Pathological Study of Primary Liver Cancer. Makuuchi M (ed). 2nd English edition, Kanehara Co., Tokyo, pp6-28, 2003.
28. Ikeda Y, Shimada M, Hasegawa H, *et al*: Prognosis of hepatocellular carcinoma with diabetes mellitus after hepatic resection. Hepatology 27: 1567-1571, 1998.
29. Poon RT, Fan ST and Wong J: Does diabetes mellitus influence the perioperative outcome or long term prognosis after resection of hepatocellular carcinoma? Am J Gastroenterol 97: 1480-1488, 2002.
30. Hui JM, Sud A, Farrell GC, *et al*: Insulin resistance is associated with chronic hepatitis C virus infection and fibrosis progression [corrected]. Gastroenterology 125: 1695-1704, 2003.
31. Qi HL, Zhang Y, Ma J, Guo P, Zhang XY and Chen HL: Insulin/protein kinase B signalling pathway upregulates metastasis-related phenotypes and molecules in H7721 human hepatocarcinoma cell line. Eur J Biochem 270: 3795-3805, 2003.
32. Goodwin PJ, Ennis M, Pritchard KI, *et al*: Fasting insulin and outcome in early-stage breast cancer: results of a prospective cohort study. J Clin Oncol 20: 42-51, 2002.
33. Hammarsten J and Hogstedt B: Hyperinsulinaemia: a prospective risk factor for lethal clinical prostate cancer. Eur J Cancer 41: 2887-2895, 2005.
34. Giovannucci E and Michaud D: The role of obesity and related metabolic disturbances in cancers of the colon, prostate, and pancreas. Gastroenterology 132: 2208-2225, 2007.
35. Lukanova A, Zeleniuch-Jacquotte A, Lundin E, *et al*: Prediagnostic levels of C-peptide, IGF-I, IGFBP -1, -2 and -3 and risk of endometrial cancer. Int J Cancer 108: 262-268, 2004.
36. Schoen RE, Weissfeld JL, Kuller LH, Thaete FL, Evans RW, Hayes RB and Rosen CJ: Insulin-like growth factor-I and insulin are associated with the presence and advancement of adenomatous polyps. Gastroenterology 129: 464-475, 2005.
37. Miyoshi Y, Funahashi T, Kihara S, Taguchi T, Tamaki Y, Matsuzawa Y and Noguchi S: Association of serum adiponectin levels with breast cancer risk. Clin Cancer Res 9: 5699-5704, 2003.
38. Cust AE, Kaaks R, Friedenreich C, *et al*: Plasma adiponectin levels and endometrial cancer risk in pre- and postmenopausal women. J Clin Endocrinol Metab 92: 255-263, 2007.
39. Baillargeon J, Platz EA, Rose DP, *et al*: Obesity, adipokines, and prostate cancer in a prospective population-based study. Cancer Epidemiol Biomarkers Prev 15: 1331-1335, 2006.
40. Ishikawa M, Kitayama J, Kazama S, Hiramatsu T, Hatano K and Nagawa H: Plasma adiponectin and gastric cancer. Clin Cancer Res 11: 466-472, 2005.
41. Wei EK, Giovannucci E, Fuchs CS, Willett WC and Mantzoros CS: Low plasma adiponectin levels and risk of colorectal cancer in men: a prospective study. J Natl Cancer Inst 97: 1688-1694, 2005.
42. Miyazaki T, Bub JD, Uzuki M and Iwamoto Y: Adiponectin activates c-Jun NH2-terminal kinase and inhibits signal transducer and activator of transcription 3. Biochem Biophys Res Commun 333: 79-87, 2005.

Hepatology Research 2009; 39: 856–864 doi: 10.1111/j.1872-034X.2009.00524.x

Original Article

Hepatitis C virus kinetics during the first phase of pegylated interferon-α-2b with ribavirin therapy in patients with living donor liver transplantation

Tatsuki Ichikawa,[1] Kazuhiko Nakao,[1] Hisamitsu Miyaaki,[1] Susumu Eguchi,[2] Mitsuhisa Takatsuki,[2] Masumi Fujimito,[1] Motohisa Akiyama,[1] Satoshi Miuma,[1] Eisuke Ozawa,[1] Hidetaka Shibata,[1] Shigeyuki Takeshita,[1] Takashi Kanematsu[2] and Katsumi Eguchi[1]

[1]*The First Department of Internal Medicine and* [2]*Department of Transplantation and Digestive Surgery, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan*

Aim: To identify the problems of pegylated interferon (PEG IFN) with ribavirin therapy against hepatitis C virus (HCV) reinfection in living donor liver transplantation (LDLT) patients. HCV kinetics during the PEG IFN with ribavirin therapy were analyzed in LDLT patients, as well as in chronic hepatitis C (CHC) patients.

Methods: The study included 80 consecutive HCV infected patients undergoing PEG IFN with ribavirin therapy (64 CHC and 16 LDLT patients) who attended the Nagasaki University Hospital for an initial visit between January 2005 and December 2007.

Results: The sustained viral response (VR) rate of the CHC group (80%) was superior to the LDLT group (22%). The viral disappearance rate of the CHC group was also superior to the LDLT group, regardless of the HCV serotype. The HCV core antigen (cAg) titer under treatment in the LDLT group was more than that of the CHC group from day 0 to week 12. The HCV cAg decrease rate of the LDLT group on the first day of treatment was less than that of the CHC group.

Conclusion: The HCV infection of a transplanted liver is more refractory to treatment than a non-transplanted liver. The low reduction HCV cAg rate on day 1 is one of the problems of the combination therapy.

Key words: chronic hepatitis C, first phase, hepatitis C virus, interferon, living donor liver transplantation

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection is widespread throughout the world. Chronic HCV infection leads to cirrhosis and hepatocellular carcinoma. Liver transplantation for HCV-related liver disease has been an option worldwide.[1] Recently, it has been shown that the prognosis for liver transplanted (LT) patients with HCV-related disease deteriorates over time,[2] thus resulting in a poorer outcome than in the non-HCV course.[3] The transplanted liver for HCV-related disease undergoes a rapidly progressive fibrosis and acute graft failure.[3,4] Consequently, anti-HCV treatment after LT is important for the prognosis. Interferon (IFN) has been recognized as the only treatment method for HCV infection. For the transplanted liver, it is known that IFN treatment improves liver fibrosis or halts the progression.[5] Recently, the combination of pegylated IFN (PEG IFN) with ribavirin was used and produced an excellent result for non-transplanted patients with HCV.[6] However, that was not the case for the HCV re-infected transplanted liver.[7] It is important that the cause of refractory HCV infection in the transplanted liver be more fully clarified. Immunosuppressant therapy, especially with glucocorticoid, has been speculated to be the cause of the refractory nature of the transplanted liver to IFN.[8,9] The cause of this is considered to be that glucocorticoid downregulated the IFN signal transduction in the hepatocytes.[8] The authors recently found that calcineurin inhibitors also inhibited IFN induced STAT-1 phosphorylation and antiviral activity in the HCV

Correspondence: Dr Tatsuki Ichikawa, The First Department of Internal Medicine, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. Email: ichikawa@net.nagasaki-u.ac.jp

Received 7 November 2008; revision 24 February 2009; accepted 5 March 2009.

replicon system.[10] Therefore, the problem of IFN signaling in the hepatocyte induced an IFN refractory condition[11] and decreased the first phase of HCV decline, which was IFN induced HCV decay during the first day of IFN treatment.[12]

In the present study, we attempted to better understand PEG IFN and ribavirin therapy by comparing patients with chronic hepatitis from HCV infection (CHC) with living donor LT (LDLT) patients. When the non-transplanted CHC patients were used as a reference against the HCV reinfected LDLT patients, we expected that the differences in the clinical data in the two groups would help to clarify the problem of IFN refractory HCV infection, and shed light on the analysis of HCV kinetics under IFN and ribavirin treatment, and to elucidate the damaged segment of the IFN induced antiviral mechanism in the LDLT condition.

PATIENTS AND METHODS

Patients

THE PRESENT RESEARCH is a prospective study. The study included 80 consecutive HCV-infected patients undergoing PEG IFN with ribavirin combination therapy (64 CHC and 16 LDLT patients) who attended the Nagasaki University Hospital for an initial visit between January 2005 and December 2007. All patients received the targeted dose of 1.5 μg/kg PEG IFN-α-2b (Pegintron; Schering-Prough K.K., Osaka, Japan) once weekly with daily ribavirin (Rebetol; Schering-Prough K.K., Osaka, Japan) for a total dose of 600 mg (bodyweight < 60 kg), 800 mg (60 kg < bodyweight < 80 kg) or 1000 mg (bodyweight > 80 kg) according to bodyweight (BW). The number of patients who were judged to have obtained a curative effect from IFN therapy was 42 in total, and 12 were LDLT patients. If the HCV-RNA had been negative in the patient serum until 12 weeks after the initiation of treatment or positive at 24 weeks, PEG IFN with ribavirin therapy was stopped at week 48. If the HCV-RNA had been negative from weeks 12 to 24, PEG IFN with ribavirin therapy was continued for 24 weeks to a predetermined 48 weeks. CHC patients were diagnosed on the basis of a persistently raised alanine aminotransferase (ALT) level and biopsy proven disease. All LDLT patients, who had undergone liver transplantation for HCV related cirrhosis at Nagasaki University Hospital from June 2002 to May 2007, had the HCV-RNA in their serum at the commencement of PEG IFN with ribavirin treatment. To prevent HCV related hepatitis after liver transplantation, pre-emptive therapy using IFN is the strategy used at the Nagasaki University Hospital. After the recovery of the general condition without ascites and icterus after transplantation, and establishment of the diagnosis using the liver biopsy, PEG IFN with ribavirin therapy was started. The interval between LDLT and IFN treatment was a mean of 281 days (range 16–989 days). Tacrolimus (Astellas, Tokyo, Japan), an immunosuppressive agent, was used together with steroids for all LDLT patients as the induction therapy. When IFN therapy was commenced, tacrolimus was switched to cyclosporin (Novartis, Tokyo, Japan) in 12/16 cases. A percutaneous liver biopsy assisted by ultrasonography was carried out in all cases. Liver histology was evaluated according to the degree of fibrosis and necroinflammatory activity.[13] The extent of fibrosis (staging) was classified as follows: F1 (periportal expansion), F2 (portoportal septa), F3 (portocentral linkage or bridging fibrosis) and F4 (cirrhosis). The necroinflammatory activity (grading) was classified as follows: A1 (mild), A2 (moderate) and A3 (severe). Liver biopsy specimens were fixed in 10% formalin, embedded in paraffin, cut to a thickness of 4 μm, and subjected to hematoxylin–eosin and Azan–Mallory staining.

Hepatitis C virus kinetics assessment

We compared the HCV viral load in both groups, determined by the HCV core antigen (cAg), at baseline (D0), day 1 (D1), week 1 (W1), week 2 (W2), week 4 (W4), week 8 (W8), week 12 (W12), week 24 (W24) and week 48 (W48). The HCV viral serotype (ST) and HCV cAg were determined using available kits. In this assay, HCV serotypes 1 and 2 correspond to genotypes 1 and 2 of Simmonds' classification,[14] respectively. The HCV cAg correlates with HCV-RNA by quantitative PCR.[15] HCV cAg was measured at the indicated times and HCV-RNA qualitative PCR, the amplicor monitor method, was used after the level was under the detection range of HCV cAg in every month. In the present study, we proposed the calculation of the decreased HCV viral load during PEG IFN with ribavirin treatment and set as follows: a negative HCV cAg was 20 fmol/L and a negative HCV-RNA qualitative PCR was 1 fmol/L.

Clinical and laboratory measurements

The body mass index (BMI) was calculated as weight (kg) divided by the square of height (m). Subjects fasted overnight before blood samples were obtained. Venous plasma glucose was measured with an automated analyzer, and basal serum insulin was measured using a standard radioimmunoassay. The index of insulin

resistance and β-cell function was calculated using the fasting value of plasma glucose (we excluded the patients with greater than 130 mg/dL), and the serum insulin level according to the homeostasis model assessment (HOMA) method. HOMA-IR, an insulin resistance marker, is calculated as follows: fasting plasma glucose × fasting insulin/405. HOMA-β, a β-cell function marker, was calculated as follows: 360 × fasting insulin/(fasting plasma glucose-63).[16] White blood cell, red blood cell, platelet, hemoglobin A1c, ALT, aspartate aminotransferase (AST), γ-GTP, total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL), high density lipoprotein (HDL), free fatty acid (FFA), and ferritin were determined by standard hematometry and laboratory techniques.

Statistical analysis

The data were processed on a personal computer and analyzed using StatView 5.0 (SAS Institute, Cary, NC, USA). Differences between groups were analyzed by Mann–Whitney *U*-test and Pearson χ^2-test. All data in the text and tables are shown as means, unless otherwise indicated. The statistical analysis of the HCV-RNA disappearance rate was by the Kaplan–Meier method with Wilcoxon assay. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Differences of patient characteristics

FIRST, THE PRETREATMENT clinical and laboratory characteristics were compared with All-CHC and All-LDLT patients (Table 1). The BW and BMI in the All-CHC group were higher than that of the All-LDLT group. Therefore, the levels of PEG IFN dose per BW and ribavirin dose per BW were even, but the levels of PEG IFN dose and ribavirin dose in the all LDLT group were lower than in the All-CHC group. The HCV viral load in the all LDLT group was greater than that in the All-CHC group and serotype 1 was the majority in the All-LDLT group. In hematology and laboratory data, the red blood cell count and hemoglobin in the All-LDLT group was lower than that of the All-CHC group, and the FFA level was higher in the All-LDLT group. In the histological examination, fibrosis is more advanced in the All-CHC group than in the All-LDLT group. There was the tendency toward higher levels of fasting plasma glucose and lower levels of HOMA-β in the All-LDLT group than in the All-CHC group. Next, we targeted the serotype 1 and a high HCV titer (ST1H group) above 100 KIU/L by the qualitative PCR method or 300 fmol/L of the cAg assay. These were examined in the same way (Table 2). The ST1H group might have shown the same result as the All group, except the levels of fasting plasma glucose and HOMA-β did not differ with ST1H-CHC and ST1H-LDLT. The mean value of fasting plasma glucose (FPG) was higher than the normal range in the LDLT group. The discontinuance rates of treatment were almost equal, 19 cases (29.7%) and 4 cases (25%) in All-CHC and All-LDLT, respectively. The reasons for discontinuance were adverse effects in All-LDLT patients and the refractory nature of viral response in two All-CHC patients.

The HCV infection in the LDLT group is more obstinate than in the CHC group

The response rate and cure rate of PEG IFN with ribavirin therapy were compared with both groups (Table 2A, All group and B, ST1H group). The HCV response rate to treatment, viral response (VR), was determined by the disappearance of HCV-RNA or by the decline of HCV cAg to less than 1/100 before treatment. The cure rate, sustained viral response rate (SVR), was determined by a negative HCV-RNA by qualitative PCR method at 6 months post-termination of treatment. The VR rate at 8 and 12 weeks, but not at 4 weeks, and the PP-SVR in the LDLT group (Table 3A,B) was worse than that in the CHC group. Non-viral responders, who did not achieve HCV-RNA negativity during the treatment, did not show statistical significance in either SG1H group (Table 3B). As a result, we calculated the prediction of the lack of SVR by non-viral response in the LDLT group. The sensitivity, specificity, positive predictive values and negative predictive value were 1, 0, 0.917 and the acalculia for null viral responders at 24 h, 0.7, 1, 1 and 0.25 at 4 weeks, 0.6, 1, 1 and 0.2 at 8 weeks and 0.6, 1, 1 and 0.2 at 12 weeks, respectively.

The disappearance rate of HCV-RNA was evaluated by the Kaplan–Meier method (Fig. 1 ST1H group). The disappearance rate in the LDLT group was statistically lower than the CHC group. Before 14 weeks after the initiation of treatment, the HCV-RNA disappearance case was not apparent in the ST1H group (Fig. 1).

The decline of HCV load, especially early phase, is blocked in the LDLT group

For the analysis of viral kinetics, we evaluated the decline of the HCV load and the decline rate after treatment with particular emphasis of the early phase of treatment, including D1-W12. In the ST1H group (Fig. 2), the decreased rate on D1 in the LDLT group was

Table 1 Difference of characteristics between all chronic hepatitis C cases and all living donor liver transplantation cases

Characteristics	All-CHC ($n = 64$)	All-LDLT ($n = 16$)	*P*-value
Age (years)	58 ± 10.8	58.8 ± 4.62	NS
Sex (male : female)	36:28	7:9	NS
Height (m)	1.60 ± 0.098	1.583 ± 0.010	NS
Bodyweight (kg)	61.0 ± 11.0	54.8 ± 8.52	0.025
Body mass index	23.6 ± 2.94	21.8 ± 2.30	0.022
PEG IFN dose (μg)	80.1 ± 18.7	71.9 ± 33.5	0.035
PEG IFN/BW	1.31 ± 0.304	1.35 ± 0.708	NS
Ribavirin dose (mg)	621.9 ± 151.7	525 ± 100	0.030
Ribavirin/BW	10.2 ± 2.23	9.72 ± 2.04	NS
Serotype (1:2)	45:17	15:1	0.081
HCV cAg (fmol/L)	5773 ± 5609	23144 ± 21059	0.001
WBC (/μL)	5006.3 ± 1335	5918.8 ± 2439	NS
RBC (10^4/μL)	445 ± 41.1	350 ± 56.7	< 0.0001
Hemoglobin (g/dL)	13.8 ± 1.06	10.9 ± 1.85	< 0.0001
Platelet (10^4/μL)	16.4 ± 4.48	18.5 ± 10.6	NS
AST (U/L)	62.9 ± 35	64.3 ± 37.2	NS
ALT (U/L)	85 ± 53.0	89.9 ± 57.1	NS
γ-GTP (U/L)	62.1 ± 56.5	138.9 ± 129.1	0.013
Ferritin (ng/dL)	218 ± 216	254 ± 259	NS
TC (mg/dL)	169.8 ± 26.6	167.3 ± 38.8	NS
TG (mg/dL)	105.3 ± 46.8	122.8 ± 44.8	0.069
HDL (mg/dL)	45.2 ± 11.9	46.6 ± 14.9	NS
LDL (mg/dL)	97.3 ± 24.3	88.8 ± 26.7	NS
FFA (mEq/L)	0.492 ± 0.261	0.686 ± 0.299	0.019
FPG (mg/dL)	91.9 ± 15.4	125.1 ± 56.9	0.090
Insulin (mIU/L)	9.16 ± 5.1	8.34 ± 5.16	NS
HOMA-IR	2.08 ± 1.22	1.75 ± 1.42	NS
HOMA-β	135.4 ± 86.2	89.7 ± 86.9	0.075
Fibrosis	1.86 ± 1.18	0.875 ± 0.806	0.004
Activity	1.03 ± 0.48	1.31 ± 0.48	0.067

Data are shown as the means ± standard deviation and values, with statistical analysis calculated by Mann–Whitney *U*-test for means and Pearson's χ^2-test for values.

Normal values in laboratory tests: ALT (IU/L), 5–40; AST (IU/L), 10–40; γ-GTP (IU/L), < 70 in males, < 30 in females; TC (mg/dL), 150–219; TG (mg/dL), 50–149; FFA (mEq/L), 0.14–0.85; LDL (mg/dL), 70–139; HDL (mg/dL), 40–86 in male, 40–96 in female; hemoglobin (g/dL), 13.5–17.6 in male, 11.3–15.2 in female; WBC (/μL), 3900–9800 in males, 3500–9100 in females; RBC (10^4/μL), 427–570 in males, 376–500 in females; ferritin (mg/dL), 27–320 in males, 3.4–89 in females; platelet (10^4/μL), 13.1–36.2 in males, 13–36.9 in females; insulin (IU/L), 3.06–16.9; FPG (mg/L), 70–109. HOMA-IR, HOMA-β, and BMI are described in the text.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHC, chronic hepatitis C; FFA, free fatty acid; FPG, fasting plasma glucose; HCV cAg, hepatitis C virus core antigen; HDL, high density lipoprotein; HOMA, homeostasis model assessment; LDL, low density lipoprotein; LDLT, living donor liver transplantation; PEG IFN, pegylated interferon; RBC, red blood cell count; TC, total cholesterol; TG, triglyceride; WBC, white blood cell count.

statistically lower than CHC (Fig. 2b) and the viral load of the LDLT group was larger than that in CHC from D0 to W12 (Fig. 2a). The decreased rate at the indicated time without D1 and W12 was not the difference between CHC and LDLT (Fig. 2b). We next analyzed the SG1H-group that matched the pre-treatment HCV cAg titer (Fig. 3). In a similar fashion to Figure 2, the viral load of the matched LDLT group was larger than that of the matched CHC from D1 to W12 (Fig. 3a) and the decreased rate of the matched LDLT group was lower than that of the matched CHC at D1, W2 and W4 (Fig. 3b).

DISCUSSION

IN THE PRESENT prospective study, we compared CHC and LDLT patients treated with PEG IFN and ribavirin for HCV infection. BMI, HCV cAg, red blood

Table 2 Difference of characteristics of serotype 1 and high virus titer between chronic hepatitis C patients and living donor liver transplantation patients

Characteristics	ST1H-CHC ($n = 42$)	ST1H-LDLT ($n = 15$)	*P*-value
Age (years)	58.5 ± 10.8	58.8 ± 4.78	NS
Sex (male : female)	22:20	6:9	NS
Height (m)	1.60 ± 0.10	1.566 ± 0.081	NS
Bodyweight (kg)	61.8 ± 12.1	53.8 ± 7.69	0.02
Body mass index	24.0 ± 2.78	21.9 ± 2.37	0.012
PEG IFN dose (μg)	81.4 ± 19.5	73.3 ± 34.2	0.052
PEG IFN/BW	1.33 ± 0.269	1.39 ± 0.711	NS
Ribavirin dose (mg)	642.8 ± 150.0	520 ± 101.4	0.011
Ribavirin/BW	10.5 ± 2.13	9.80 ± 2.08	NS
HCV cAg (fmol/L)	6969 ± 5281	24674 ± 20856	0.003
WBC (/μL)	5019.0 ± 1294	6033.8 ± 2479	NS
RBC (10^4/μL)	444 ± 40.1	351 ± 58.6	< 0.0001
Hemoglobin (g/dL)	13.9 ± 1.10	10.8 ± 1.88	< 0.0001
Platelet (10^4/μL)	16.7 ± 4.68	18.9 ± 10.8	NS
AST (U/L)	62.1 ± 31.6	64.2 ± 38.5	NS
ALT (U/L)	84.5 ± 51.8	88.0 ± 58.6	NS
γ-GTP (U/L)	64.0 ± 61.7	113.6 ± 83.1	0.036
Ferritin (ng/dL)	206 ± 164.8	204.5 ± 188.4	NS
TC (mg/dL)	172.6 ± 25.7	165.3 ± 39.2	NS
TG (mg/dL)	108.2 ± 52.2	122.9 ± 46.4	NS
HDL (mg/dL)	46.5 ± 11.9	45.4 ± 14.8	NS
LDL (mg/dL)	97.7 ± 25.4	88.6 ± 27.8	NS
FFA (mEq/L)	0.514 ± 0.251	0.693 ± 0.310	0.049
FPG (mg/dL)	92.4 ± 16.4	123.7 ± 58.6	NS
Insulin (mIU/L)	9.06 ± 5.5	8.34 ± 5.16	NS
HOMA-IR	2.07 ± 1.31	1.86 ± 1.38	NS
HOMA-b	128.0 ± 76.2	95.7 ± 86.5	NS
Fibrosis	1.92 ± 1.19	0.933 ± 0.799	0.008
Activity	1.08 ± 0.474	1.33 ± 0.488	0.098

Data are shown as the means ± standard deviation and values, with statistical analysis calculated by Mann–Whitney *U*-test for means and Pearson's χ^2-test for values.

Normal values in laboratory tests are same as in Table 1.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHC, chronic hepatitis C; FFA, free fatty acid; FPG, fasting plasma glucose; HCV cAg, hepatitis C virus core antigen; HDL, high density lipoprotein; HOMA, homeostasis model assessment; LDL, low density lipoprotein; LDLT, living donor liver transplantation; PEG IFN, pegylated interferon; RBC, red blood cell count; TC, total cholesterol; TG, triglyceride; WBC, white blood cell count.

cell, γ-GTP, FFA and liver fibrosis in the pretreatment clinical characteristics were different in both groups (Tables 1,2). The VR rate of the CHC group was superior to that of the LDLT group, and the SVR by per-protocol analysis was also similar in result to the VR (Table 3). The viral disappearance rate of the CHC group was superior to the LDLT group, regardless of the HCV serotype (Fig. 1). The HCV cAg titer under the treatment in the LDLT group was more than that of the CHC group from D0 to W12 (Figs 2a,3a) and the HCV cAg decrease rate of the LDLT group at the D1 was less than that of the CHC group (Figs 2b,3b). We showed that the reinfected HCV to the graft liver was more refractory than the non-transplanted CHC. The PEG IFN and ribavirin dose per BW was an equal dose in both groups. However, it was difficult to determine the pretreatment predictive factors for the LDLT cases, because only one case showed SVR in the LDLT group. Thus, we considered that the difference of the pretreatment clinical characteristics in both groups might be related to the refractory HCV infection.

The pretreated HCV cAg titer is known to be the principal factor for IFN resistance. For CHC and LDLT patients, a high HCV-RNA titer in the pretreatment sera

Table 3 Result of pegylated interferon-α-2b plus ribavirin therapy

A. All cases			
Term	All-CHC	All-LDLT	*P*-value
Viral response 4 weeks	40/60 (67%)	5/12 (42%)	NS
Viral response 8 weeks	47/55 (85%)	6/12 (50%)	0.011
Viral response 12 weeks	43/48 (90%)	6/12 (50%)	0.003
Sustained viral response: ITT	20/42 (45%)	2/12 (20%)	0.054
Sustained viral response: PP	20/28 (80%)	2/9 (22%)	0.008
B. Serotype 1 and high virus titer cases			
Term	ST1H-CHC	ST1H-LDLT	*P*-value
Viral response 4 weeks	24/40 (67%)	5/11 (45%)	NS
Viral response 8 weeks	30/36 (83%)	5/11 (45%)	0.012
Viral response 12 weeks	25/29 (86%)	5/11 (45%)	0.008
Sustained viral response: ITT	8/27 (30%)	1/11 (8%)	NS
Sustained viral response: PP	8/15 (53%)	1/9 (11%)	0.029
Non-virological response: ITT	11/27 (41%)	5/11 (45%)	NS
Non-virological response: PP	4/15 (27%)	4/9 (44%)	NS

Data are shown as relevant numbers/target case numbers (percentage of relevant numbers) with statistical analysis using Pearson's χ^2-test for numbers.
CHC, chronic hepatitis C; ITT, intention to treatment analysis; LDLT, living donor liver transplantation; PP, per-protocol analysis.

is associated with non-responder status for IFN treatment.[7,17] In the LDLT condition, the HCV-RNA titer was rapidly increased after immediately decreasing at transplant and the viral load after several weeks post-LDLT exceeded the value of pre-LDLT.[18] The HCV-RNA titer increased rapidly in patients receiving corticosteroids as part of the immunosuppressant regimen.[18,19] We have speculated that the massive amount of HCV, caused by immunosuppressant therapy after the LDLT, was part of the reason for the IFN refractory status. However, comparisons with the pretreated HCV cAg matched groups (Fig. 3) showed the existence of an important factor other than the pretreatment viral load. It will, therefore, be necessary to analyze this problem by evaluating many factors, for example immunosuppressants[10] and regeneration, in the future.

A high level of γ-GTP was also known to be an important factor for IFN treatment.[7,17] Usually, high levels of γ-GTP and FFA have been linked to insulin resistance.[20,21] Therefore, insulin resistance in the liver is assumed in the condition of IFN resistance. However, the LDLT group had the normal range of HOMA-IR,[16] which was lower than that of the CHC group (Tables 1,2). The HCV infection after liver transplantation is associated with insulin resistance.[22] Immunosuppressants, especially corticosteroids, induced insulin resistance.[23] In the present study, the LDLT group had a disturbance of insulin secretion

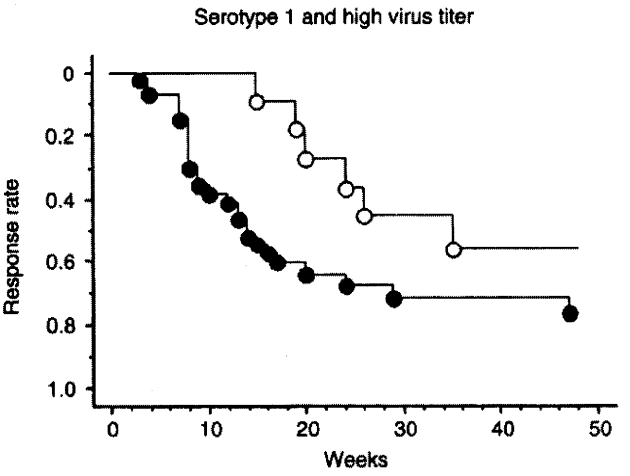
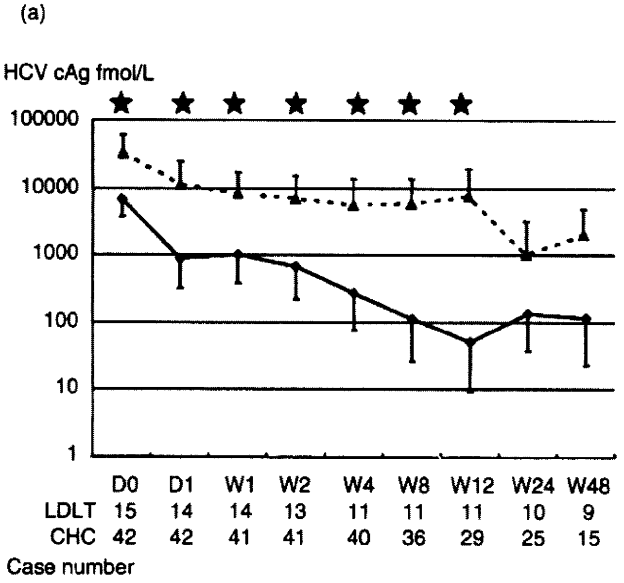


Figure 1 The difference in the hepatitis C ribonucleic acid (HCV-RNA) disappearance rate between the chronic hepatitis C (CHC) group and living donated liver transplantation (LDLT) group during 48 weeks of treatment. HCV-RNA was evaluated by the qualitative PCR method. The disappearance rate was calculated as follows: serum HCV-RNA disappearance case number/all cases in indicated time. The statistical analysis was carried out using the Kaplan–Meier method with the Wilcoxon assay. ST1H group was plotted as the HCV-RNA disappearance line between the white circle of the LDLT group and the black circle of the CHC group. In all cases and the ST1H group, the disappearance rate was statistically significant between the CHC group and the LDLT group ($P < 0.05$).



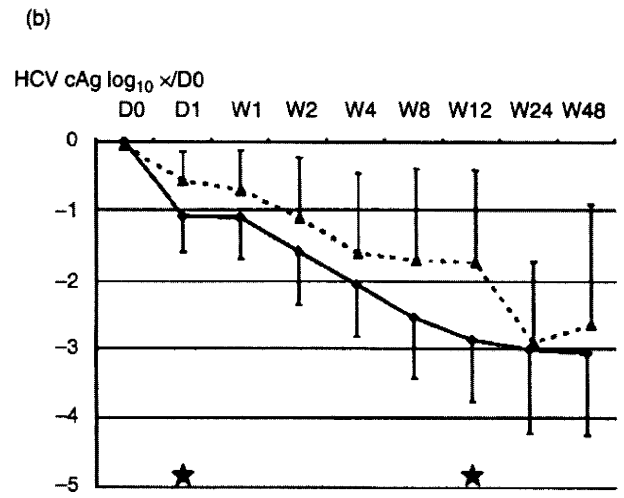
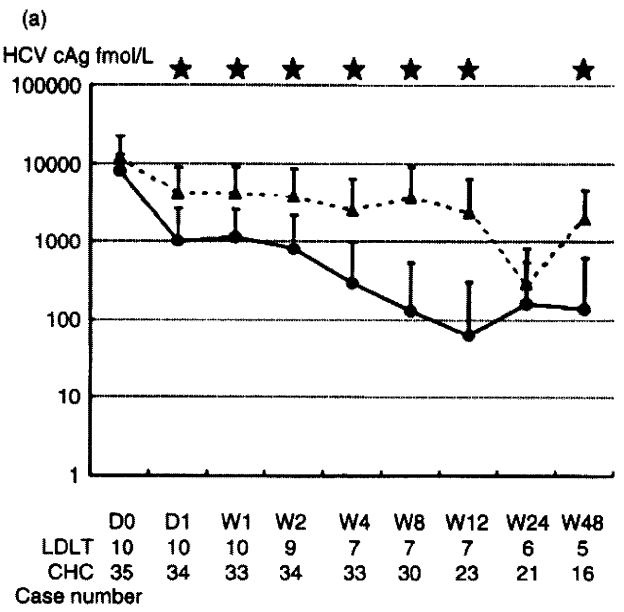


Figure 2 Comparison of viral kinetics between the SG1H-chronic hepatitis C (CHC) group and the SG1H-living donor liver transplantation (LDLT) group during the 48 weeks of treatment. (a) The hepatitis C virus core antigen (HCV cAg) load and (b) reduction rates were plotted by a straight line (SG1H-CHC group), and dotted line (SG1H-LDLT group). The error bar represented the standard deviation. On the *y*-axis, D0 is pretreatment, D1 and WX is time post-treatment day 1 and week X, respectively. The reduction rate was calculated as follows: $\log_{10}$HCV cAg load in indicated time/in D0. HCV cAg titer at the indicated time between SG1H-CHC and SG1H-LDLT were compared. The asterisk mark indicates a significant difference, $P < 0.05$, calculated by Mann–Whitney *U*-test.



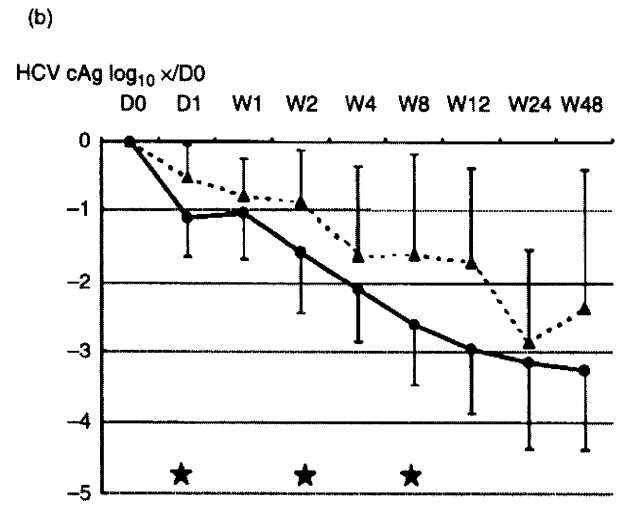


Figure 3 Comparison of viral kinetics between matched pre-treatment hepatitis C virus core antigen (HCV cAg) ST1H-chronic hepatitis C (CHC) group and ST1H- living donor liver transplantation (LDLT) group during 48 weeks of treatment. (a) HCV cAg load and (b) reduction rate were plotted by a straight line (matched SG1H-CHC group) and dotted line (matched SG1H-LDLT group). The error bar represents the standard deviation. The asterisk mark is the significant difference, $P < 0.05$, calculated by Mann–Whitney *U*-test.

rather than insulin resistance and high levels of FPG might be caused by the disturbance of insulin secretion. Therefore, further study is necessary to clarify the relationship between the glucose metabolism and the IFN resistance in LDLT patients. The levels of γ-GTP rise at cholestatic conditions. It was reported that the presence

of a cholestatic profile is associated with an adverse response to IFN treatment in LT.[7] A cholestatic profile provoked the TH2-like lymphocyte response.[19] The authors have previously reported that IL-10, representative of TH2 cytokine, inhibits IFN signaling through an inducible suppressor of cytokine signaling.[24] The high levels of FFA were induced by a catabolic state, such as cirrhosis, and were not fully recovered after LDLT. As a result, the levels of FFA reflected a continuous catabolic state at the beginning of IFN treatment. FFA can induce oxidative stress in various cells,[25,26] and inhibit the IFN induced antiviral gene induction through the inactivation of Jak-1 and Tyk-2.[27] Therefore, we are speculating that high levels of γ-GTP and FFA in the LDLT group have the ability to inhibit IFN signaling as much as in the CHC patients.

We are paying attention to the viral decline of D1/D0 (Figs 2b,3b). The decreased rate of D1 is named as the first phase of HCV decline and is the predictor of SVR.[28,29] The first phase influenced the second phase, which is the decline of HCV after D2.[28] The IFN induced antiviral gene products were considered to be very important for antiviral activity.[11] The expressions of the IFN stimulating genes (ISG) were associated with the early phase of the decline[11] and it was reported that the lack of ISG caused early liver fibrosis in the LT patients with HCV.[30] In the LDLT group, the reduced HCV cAg decreased the rate of D1 and this might be part of the cause of being refractory to IFN. We speculate that an IFN signaling disturbance, related to high levels of γ-GTP and FFA, might have triggered the adverse effect to the HCV cAg decreased rate of D1.

In summary, it became clear that the viral response and SVR is worse in the LDLT group. The first phase of viral decay, the decreased rate of D1/D0, also declined in the LDLT group. High levels of γ-GTP and FFA in the pretreatment sera might also be related to IFN-signaling damage in hepatocytes. At the initiation of pre-emptive therapy, HCV had also been increasing in the graft liver and the catabolic status of energy did not recover for the relatively small size of the graft liver. When beginning treatment for an HCV infection after LT, we should carefully take into account the timing of IFN initiation, in addition to the types of immunosuppressants used.

REFERENCES

1 Perz JF, Armstrong GL, Farrington LA *et al.* The contributions of hepatitis b virus and hepatitis c virus infections to cirrhosis and primary liver cancer worldwide. *J Hepatol* 2006; **45**: 529–38.

2 Forman LM, Lewis JD, Berlin JA *et al.* The association between hepatitis C infection and survival after orthotopic liver transplantation. *Gastroenterology* 2002; **122**: 889–96.

3 Berenguer M, Prieto M, San Juan F *et al.* Contribution of donor age to the recent decrease in patient survival among HCV-infected liver transplant recipients. *Hepatology* 2002; **36**: 202–10.

4 Berenguer M, Ferrell L, Watson J *et al.* HCV-related fibrosis progression following liver transplantation: increase in recent years. *J Hepatol* 2000; **32**: 673–84.

5 Carrion JA, Navasa M, Garcia-Retortillo M *et al.* Efficacy of antiviral therapy on hepatitis C recurrence after liver transplantation: a randomized controlled study. *Gastroenterology* 2007; **132**: 1746–56.

6 Davis GL, Wong JB, McHutchison JG *et al.* Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. *Hepatology* 2003; **38**: 645–52.

7 Fernandez I, Meneu JC, Colina F *et al.* Clinical and histological efficacy of pegylated interferon and ribavirin therapy of recurrent hepatitis C after liver transplantation. *Liver Transpl* 2006; **12**: 1805–12.

8 Hu X, Li WP, Meng C *et al.* Inhibition of IFN-gamma signaling by glucocorticoids. *J Immunol* 2003; **170**: 4833–9.

9 Boor PP, Metselaar HJ, Mancham S *et al.* Prednisolone suppresses the function and promotes apoptosis of plasmacytoid dendritic cells. *Am J Transpl* 2006; **6**: 2332–41.

10 Hirano K, Ichikawa T, Nakao K *et al.* Differential effects of calcineurin inhibitors, tacrolimus and cyclosporine A, on interferon induced anti-viral protein in human hepatocyte cell. *Liver Transpl* 2008; **14**: 295–301.

11 Feld JJ, Nanda S, Huang Y *et al.* Hepatic gene expression during treatment with peginterferon and ribavirin: Identifying molecular pathways for treatment response. *Hepatology* 2007; **46**: 1548–63.

12 Neumann AU, Lam NP, Dahari H *et al.* Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. *Science* 1998; **282**: 103–7.

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

14 Tanaka T, Tsukiyama-Kohara K, Yamaguchi K *et al.* Significance of specific antibody assay for genotyping of hepatitis C virus. *Hepatology* 1994; **19**: 1347–53.

15 Gonzalez V, Padilla E, Diago M *et al.* Clinical usefulness of total hepatitis C virus core antigen quantification to monitor the response to treatment with peginterferon alpha-2a plus ribavirin*. *J Viral Hepat* 2005; **12**: 481–7.

16 Taura N, Ichikawa T, Hamasaki K *et al.* Association between liver fibrosis and insulin sensitivity in chronic hepatitis C patients. *Am J Gastroenterol* 2006; **101**: 2752–9.

17 Taliani G, Gemignani G, Ferrari C *et al.* Pegylated interferon alfa-2b plus ribavirin in the retreatment of interferon-ribavirin nonresponder patients. *Gastroenterology* 2006; **130**: 1098–106.

18 Garcia-Retortillo M, Forns X, Feliu A *et al.* Hepatitis C virus kinetics during and immediately after liver transplantation. *Hepatology* 2002; **35**: 680–7.
19 McCaughan GW, Zekry A. Mechanisms of HCV reinfection and allograft damage after liver transplantation. *J Hepatol* 2004; **40**: 368–74.
20 Mook S, Halkes CJ C, Bilecen S *et al.* In vivo regulation of plasma free fatty acids in insulin resistance. *Metabolism* 2004; **53**: 1197–201.
21 Kronenberger B, Herrmann E, Micol F *et al.* Viral kinetics during antiviral therapy in patients with chronic hepatitis C and persistently normal ALT levels. *Hepatology* 2004; **40**: 1442–9.
22 Delgado-Borrego A, Casson D, Schoenfeld D *et al.* Hepatitis C virus is independently associated with increased insulin resistance after liver transplantation. *Transplantation* 2004; **77**: 703–10.
23 Bloom RD, Lake JR. Emerging issues in hepatitis C virus-positive liver and kidney transplant recipients. *Am J Transpl* 2006; **6**: 2232–7.
24 Ichikawa T, Nakao K, Nakata K *et al.* Involvement of IL-1beta and IL-10 in IFN-alpha-mediated antiviral gene induction in human hepatoma cells. *Biochem Biophys Res Commun* 2002; **294**: 414–22.
25 Oprescu AI, Bikopoulos G, Naassan A *et al.* Free fatty acid-induced reduction in glucose-stimulated insulin secretion: evidence for a role of oxidative stress in vitro and in vivo. *Diabetes* 2007; **56**: 2927–37.
26 Tripathy D, Mohanty P, Dhindsa S *et al.* Elevation of free fatty acids induces inflammation and impairs vascular reactivity in healthy subjects. *Diabetes* 2003; **52**: 2882–7.
27 Di Bona D, Cippitelli M, Fionda C *et al.* Oxidative stress inhibits IFN-alpha-induced antiviral gene expression by blocking the JAK-STAT pathway. *J Hepatol* 2006; **45**: 271–9.
28 Layden JE, Layden TJ, Reddy KR *et al.* First phase viral kinetic parameters as predictors of treatment response and their influence on the second phase viral decline. *J Viral Hepat* 2002; **9**: 340–5.
29 Boulestin A, Kamar N, Sandres-Saune K *et al.* Twenty-four hour kinetics of hepatitis C virus and antiviral effect of alpha-interferon. *J Med Virol* 2006; **78**: 365–71.
30 Smith MW, Walters KA, Korth MJ *et al.* Gene expression patterns that correlate with hepatitis C and early progression to fibrosis in liver transplant recipients. *Gastroenterology* 2006; **130**: 179–87.

Liver International ISSN 1478-3223

CLINICAL STUDIES

Study of liver-targeted regulatory T cells in hepatitis B and C virus in chronically infected patients

Hisamitsu Miyaaki[1]*, Huijuan Zhou[1,2]*, Tatsuki Ichikawa[1], Kazuhiko Nakao[1], Hidetaka Shibata[1], Shigeyuki Takeshita[1], Motohisa Akiyama[1], Eisuke Ozawa[1], Satoshi Miuma[1] and Katsumi Eguchi[1]

1 The First Department of Internal Medicine, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki, Japan
2 The Department of Infectious Disease, Shanghai Jiaotong University School of Medicine, 197 Rui Jin Er Road, Shanghai, China

Keywords
Foxp3 – hepatitis B virus – hepatitis C virus – regulatory T cell

Abbreviations
HBV, hepatitis B virus; HCV, hepatitis C virus; CHB, chronic hepatitis B; CHC, chronic hepatitis C; GITR, glucocorticoid-induced TNF receptor-related protein; Foxp3, forkhead box P3

Correspondence
Hisamitsu Miyaaki, MD, The First Department of Internal Medicine, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
Tel: +81 95 819 7267
Fax: +81 95 819 7267
e-mail: miyaaki-hi@umin.ac.jp

Received 8 January 2008
Accepted 18 June 2008

DOI:10.1111/j.1478-3231.2008.01842.x

Abstract
Introduction: Regulatory T cells (Tregs) play a critical role in chronic viral infections. The role of Tregs in chronic hepatitis B (CHB) and chronic hepatitis C (CHC) is unknown. This study examined the distribution and frequency of forkhead box $p3^+$ ($Foxp3^+$) Tregs in the liver tissue and compared the clinicopathological characteristics of CHB and CHC patients. *Methods:* Liver needle biopsies were obtained from 26 patients who were hepatitis B surface antigen positive and 27 patients who were hepatitis C virus antibody positive. *Results:* The ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells was similar in HBV and in HCV cases. In HBV cases, the variables that were positively associated with the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells included the serum alanine aminotransferase level ($R = 0.402$, $P = 0.025$) and the ratio of $CD8^+$ T cell plus $CD56^+$ NK cell against $CD4^+$ T cell ($R = 0.53$, $P = 0.005$). The ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells increased more in the severe activity group than in the mild activity group ($P = 0.04$). In HCV cases, the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells increased significantly in terms of the genotype2 ($P = 0.0002$) and male gender ($P = 0.04$). In addition, the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells showed a negative correlation with the ratio of $CD8^+$ T cell plus $CD56^+$ NK cell against $CD4^+$ T cell ($R = -0.508$, $P = 0.005$) and HCV viral load ($R = -0.482$, $P = 0.001$). *Conclusions:* Liver-targeted regulatory T cells present similarly in CHB and CHC, but their relationship with the effector cell population, the inflammation grade or the viral load is different between CHB and CHC.

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are global health problems and both cause chronic hepatitis, cirrhosis and hepatocellular carcinoma (1, 2). In some cases, these viruses lead to a chronic infection in the liver, but the mechanisms by which these hepatitis viruses are able to evade the immuno system are still only poorly understood.

Recent studies have focused on regulatory T cells (Tregs). Tregs are engaged in the maintenance of self-tolerance by suppressing the activation and expansion of self-reactive lymphocytes (3–5). This suppressing function may lead to chronic inflammation and/or autoimmunity (6–12). Most of these Treg markers, including CD25, CTLA-4 and glucocorticoid-induced TNF receptor-related protein (GITR), do not accurately represent CD4+ T cells with regulatory activity, and they overlap with activated T cells, which do not necessarily possess a regulatory activity (13, 14). Currently, the best indicator of the Tregs function is thought to be the intracellular expression of forkhead box P3 (Foxp3), which is also crucial for Treg development (15). Previous reports have indicated that Tregs play a role in viral persistence by suppressing the virus-specific T cell responses in a cell-to-cell contact manner (16, 17). A more detailed analysis of this cell reaction is necessary not only in the peripheral blood but also in the target organ tissue. The liver is the target organ of both HBV and HCV. This study examined the distribution and frequency of Tregs in the liver and compared the clinicopathlogical characteristics of chronic hepatitis B (CHB) and chronic hepatitis C (CHC) patients.

Patients and methods

Liver needle biopsies were obtained from 26 patients who were hepatitis B surface antigen positive (mean age: 33.3 ± 10.4, male: female = 23:3) and 27 patients who were hepatitis C positive virus antibody positive (mean age: 57.8 ± 10.6, male: female = 15:12) at Nagasaki Universities and associated hospitals.The clinical and biological parameters, including HBV viral load, of all the patients were assessed using the HBV transcription-mediated amplification method or HCV virus load by the quantitative RT-PCR method. None of the HBV cases had been administered any antiviral treatment, while 10 of 27 HCV cases received antiviral treatment.

The clinical data of the patients are summarized in Table 1. Liver biopsy tissue specimens were taken by a needle puncture for diagnostic purposes. The diagnosis of each case was

*Co-first authors.

Table 1. Clinical data of the patients

	HBV 26 cases	HCV 27 cases
Mean age (years old)	33.6 ± 10.4	57.8 ± 10.6
Male:female	23:3	15:12
ALT (IU/L)	310 ± 472	91 ± 54
AST (IU/L)	208 ± 333	67 ± 36
Plt (× 10^4/μl)	16.6 ± 5.6	16.9 ± 4.7
Inflammation grade (mild:severe)	19:7	22:5
Fibrosis stage (mild:severe)	15:11	14:13
HBV viral load LEG/ml (TMA)	6.8 ± 1.8	
HCV genotype (1:2)		21:6
Antiviral treatment (naïve:former treatment)		17:10
HCV viral load KIU/ml (RT-PCR)		1637 (5–5000)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBV, hepatitis B virus, HCV, hepatitis C virus; RT-PCR, reverse transcriptase polymerase chain reaction; TMA, transcription-mediated amplification.

independently confirmed histologically by liver pathologists according to the Japanese chronic hepatitis classification (New Inuyama classification). Mild activity was defined as A0 or A1, and severe activity was defined as A2 or A3 by the Inuyama classification. Mild fibrosis was defined as F0 or F1, and severe fibrosis was defined as F2, F3 or F4 by the Inuyama classification. All tissues were fixed in 10% neutral-buffered formalin and were then embedded in paraffin, and 4-μm-thick serial sections were cut from each paraffin block.

T cells were examined immunohistochemically with the anti-CD3 antibody (Novocastra, Newcastle, UK), regulatory T cells were examined with the anti-Foxp3 antibody (eBioscience, San Diego, CA, USA), $CD4^+$ T cells were examined with the anti-CD4 antibody (Novocastra), $CD8^+$ T cells were examined with the anti-CD8 antibody (Novocastra) and NK cells were examined with the anti-CD56 antibody (Novocastra). The number of $Foxp3^+$, $CD4^+$, $CD8^+$, $CD56^+$ and $CD3^+$ cells contained within three portal tracts selected in each specimen were counted at a magnification of × 200. To correct for differences in the size of the portal tracts, the average proportion of $FoxP3^+$ Tregs among the total number of $CD3^+$ T cells and the average ratios of $CD8^+$ T cell plus $CD56^+$ NK cells against $CD4^+$ T cells were determined.

Statistical analysis

The SPSS statistical software program (SPSS, Chicago, IL, USA) was used to assess any correlations among multiple variables. Differences between the groups were analysed using the Mann–Whitney *U* test. Correlations between the groups were determined by the Spearman analysis. A *P* value of < 0.05 was considered to be statistically significant.

Results

The population of $Foxp3^+$ regulatory T cells in the liver

In HBV and HCV cases, both $CD3^+$ and $Foxp3^+$ lymphocytes were mainly seen in the portal areas (Fig. 1). In HBV cases, the average ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells in the portal tract was 0.09 ± 0.04, and the average ratio of $CD8^+$ T cell plus $CD56^+$ NK cell against $CD4^+$ T cell was 0.93 ± 0.63, while in HCV cases, the average ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells was 0.09 ± 0.05, and the average ratio of $CD8^+$ T cell plus $CD56^+$ NK cell against $CD4^+$ T cell was 0.95 ± 0.51.

Association between the laboratory data and the frequency of $Foxp3^+$ regulatory T cells in the liver

In HBV and HCV cases, there was no significant correlation between the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells and age. In HBV cases, the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells showed no significant difference in either gender (Table 2). On the other hand, in HCV cases, the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells was significantly increased in males in comparison with females (male:female = 0.11 ± 0.05:0.07 ± 0.03, $P = 0.04$; Table 3). In HBV cases, there was a significant correlation between the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells and the serum ALT level ($R = 0.481$, $P = 0.026$; Fig. 2, Table 2). Similarly, there was a significant correlation between the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cell and AST level ($R = 0.402$, $P = 0.025$; Table 2). On the other hand, in HCV cases, there was no significant correlation between the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells and either serum AST or ALT level ($R = -0.177$, $P = 0.38$, $R = -0.127$, $P = 0.53$; Fig. 2, Table 3).

Both in HBV and in HCV cases, there was no significant correlation between the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells and the platelet count ($R = -0.10$, $P = 0.91$, $R = -0.18$, $P = 0.37$, respectively; Tables 2 and 3).

In HBV cases, there was no significant correlation between the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells and HBV DNA viral load ($R = -0.314$, $P = 0.19$; Table 2). In HCV cases, there was a significant inverse correlation between the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells and HCV viral load ($R = -0.487$, $P = 0.01$; Fig. 3a, Table 3). In addition, the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells was significantly increased in the genotype2 group in comparison with the genotype1 group (genotype1:genotype2 = 0.07 ± 0.03:0.11 ± 0.05, $P = 0.0002$; Fig. 3b, Table 3). In HCV cases, the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells showed no significant difference between the naïve group and the former antiviral treatment group (0.08 ± 0.05:0.10 ± 0.03, $P = 0.40$; Table 3).

Association between the histological findings and the frequency of $Foxp3^+$ regulatory T cells in the liver

In HBV cases, the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells was significantly increased in the severe activity group in comparison with the mild activity group (mild:severe = 0.08 ± 0.03:0.11 ± 0.04, $P = 0.04$; Fig. 4, Table 2). In HCV cases, there was no significant difference in either activity group (mild:severe = 0.100.05:0.06 ± 0.03, $P = 0.09$; Fig. 4, Table 3). There were no significant differences among the fibrosis groups in the HBV and HCV cases (mild:severe = 0.09 ± 0.04:0.10 ± 0.03, $P = 0.58$, mild: severe = 0.09 ± 0.05:0.10 ± 0.05, $P = 0.65$, respectively; Tables 2 and 3).

Association between the ratio of $Foxp3^+$ regulatory T cells in $CD3^+$ T cells and the ratio of $CD8^+$ T cells plus $CD56^+$ NK cells against $CD4^+$ T cells in the liver

In HBV cases, a positive correlation was observed between the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells and the ratio of $CD8^+$ T cells plus $CD56^+$ NK cells in $CD4^+$ T cells ($R = 0.53$, $P = 0.005$; Table 2). In contrast, in the HCV cases, a negative correlation was observed between them ($R = -0.508$, $P = 0.005$; Table 3).

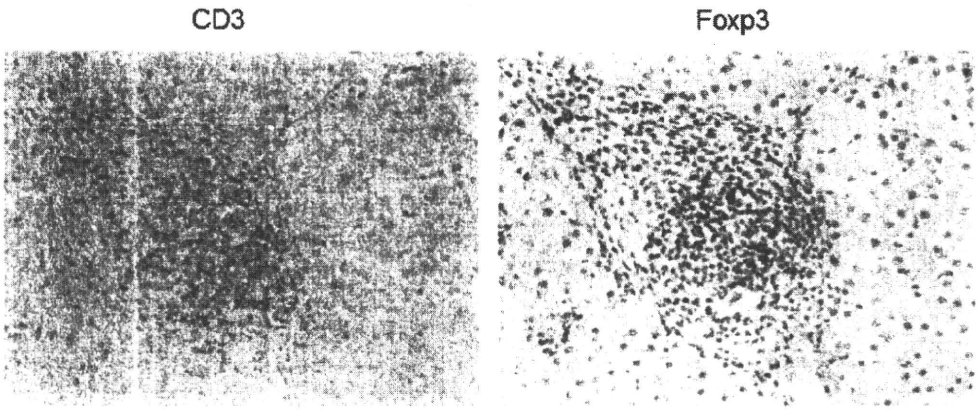


Fig. 1. Immunostaining of $CD3^+$ lymphocytes and $Foxp3^+$ Tregs in representive liver sections of patients with CHB and CHC.

Table 2. Comparison between Foxp3/CD3 and each parameter in Chronic hepatitis B

		P value*
Sex (male:female)	0.09 ± 0.04:0.09 ± 0.03	0.97
Age	$R = -0.118$	0.57
AST	$R = 0.402$	0.025
ALT	$R = 0.481$	0.026
Plt	$R = -0.10$	0.90
Grading (mild:severe)	0.08 ± 0.03:0.11 ± 0.04	0.04
Staging (mild:severe)	0.09 ± 0.04:0.10 ± 0.03	0.58
Viral load	$R = 0.314$	0.19
(CD8+CD56)/CD4†	$R = 0.53$	0.005

*Differences between the groups were analysed using the Mann–Whitney *U* test. Correlations between the groups were determined by the Spearman analysis.

†The ratio of $CD8^+$ T cell plus $CD56^+$ NK cell against $CD4^+$ T cell.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; NK, natural killer.

Table 3. Comparison between Foxp3/CD3 and each parameter in Chronic hepatitis C

		P value*
Sex (male:female)	0.11 ± 0.05:0.07 ± 0.03	0.04
Age	$R = 0.255$	0.20
AST	$R = -0.177$	0.38
ALT	$R = -0.127$	0.53
Plt	$R = -0.18$	0.37
Grading (mild:severe)	0.10 ± 0.05:0.06 ± 0.03	0.09
Staging (mild:severe)	0.09 ± 0.05:0.10 ± 0.05	0.65
Genotype (1:2)	0.07 ± 0.03:0.11 ± 0.05	0.0002
Antiviral treatment (naïve:former treatment)	0.08 ± 0.05:0.10 ± 0.03	0.40
Viral load	$R = -0.482$	0.01
(CD8+CD56)/CD4†	$R = -0.508$	0.005

*Differences between the groups were analysed using the Mann–Whitney *U* test. Correlations between the groups were determined by the Spearman analysis.

†The ratio of $CD8^+$ T cell plus $CD56^+$ NK cell against $CD4^+$ T cell.

ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Discussion

Both HBV and HCV infect the liver and cause acute and chronic liver disease (1, 2). However, the mechanism of persistent infection of these viruses is not clear. Some studies have reported that $CD4^+$ $CD25^{high}$ regulatory T cells (Tregs) play a critical role in persistent virus infection (18–20). Recently, forkhead box P3 (Foxp3) was detected as a specific marker of Tregs (12). The present study examined the distribution of $Foxp3^+$ Tregs in the liver and clarified the role of Tregs in chronic viral hepatitis.

A previous study showed that the normal human liver does not display a significant population of Tregs. This may mean that the tolerance of the liver is not primarily maintained by Tregs. In this study, $Foxp3^+$ regulatory T lymphocyte populations in the liver constitute 9% of $CD3^+$ T cell in both HBV and HCV cases. Other studies have shown that Tregs accumulate and expand locally at the site of infection where they exert their suppressive activity (21–23). This suggests that Tregs may be a key factor in the pathogenesis of chronic viral hepatitis.

In HBV cases, the variables that were significantly associated with the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells included the transaminase level (AST and ALT) and pathological grading. These factors are characteristics of the degree of inflammation in the liver. Indeed, the ratio of CD8/CD3 correlated significantly with the transaminase level (AST and ALT) in HBV cases ($r = 0.428$, $P = 0.029$, $r = 0.495$, $P = 0.010$; data not shown). These results indicated that, when severe hepatitis occurred in CHB, the number of $Foxp3^+$ Treg increases. The ratio of $CD8^+$ T cell+$CD56^+$ NK cell against $CD4^+$ cells significantly correlated with the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells. These observations suggest that Tregs may migrate into the liver during the necroinflammatory reactions induced by the virus-specific effector T cells. If Treg acts too late, the unlimited effector T cells may lead to excessive destruction and the death of hepatocyte. Therefore, it is possible that Tregs suppress the excessive activity of effector T cells in severe hepatitis by HBV.

In contrast, in HCV cases, no correlation was found between alanine aminotransferase or pathological grading and the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells. Moreover, the ratio of $CD8^+$ T cell+$CD56^+$ NK cell against $CD4^+$ cells showed a significant inverse correlation with the ratio of $Foxp3^+$ Tregs in $CD3^+$ T cells. In daily medical examination, the patients rarely experienced acute exacerbation of chronic hepatitis in HCV cases.

Liver International (2009)

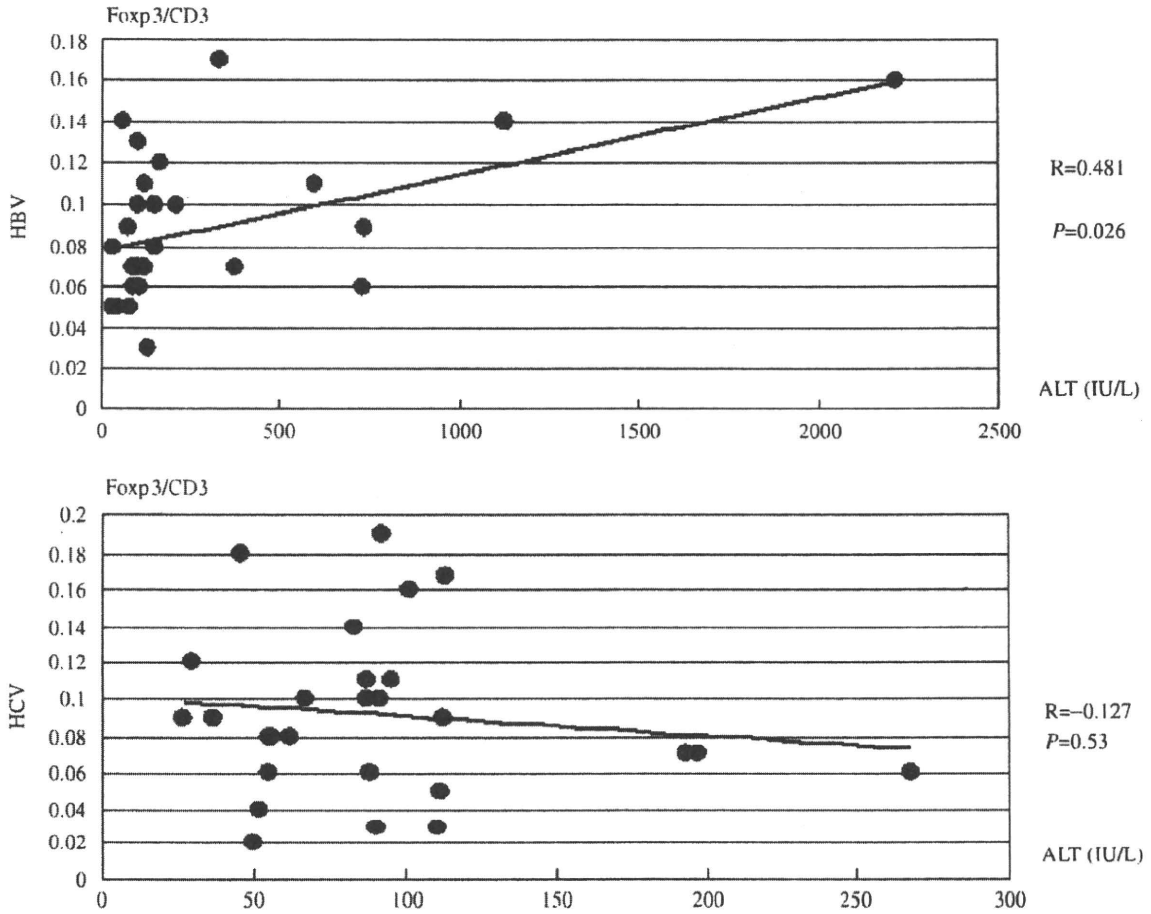


Fig. 2. Comparison between the ratio of Foxp3$^+$ Tregs in CD3$^+$ T cell and serum alanine aminotransferase level in chronic hepatitis B (CHB) and in chronic hepatitis C. A significantly positive correlation was observed between the frequency of Foxp3$^+$ Treg and the serum alanine aminotransferase level in chronic hepatitis B by the Spearman analysis ($R = 0.402$, $P = 0.025$).

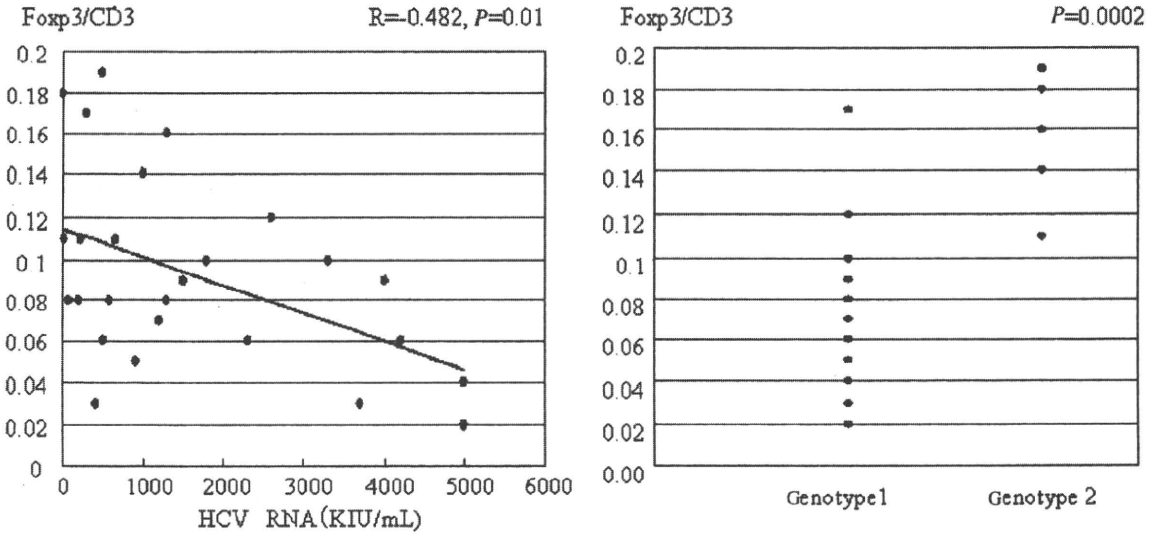


Fig. 3. (a) Comparison between the ratio of Foxp3$^+$ Tregs in CD3$^+$ T cells and HCV RNA (KIU/ml). A significantly negative correlation was found between the frequency of Foxp3$^+$ Tregs and HCV core protein by the Spearman analysis ($R = -0.482$, $P = 0.01$), (b) Comparison between the ratio of Foxp3$^+$ Tregs in CD3$^+$ T cells and HCV genotype. A significant difference was observed in the genotype by the Mann–Whitney U test ($P = 0.0002$).

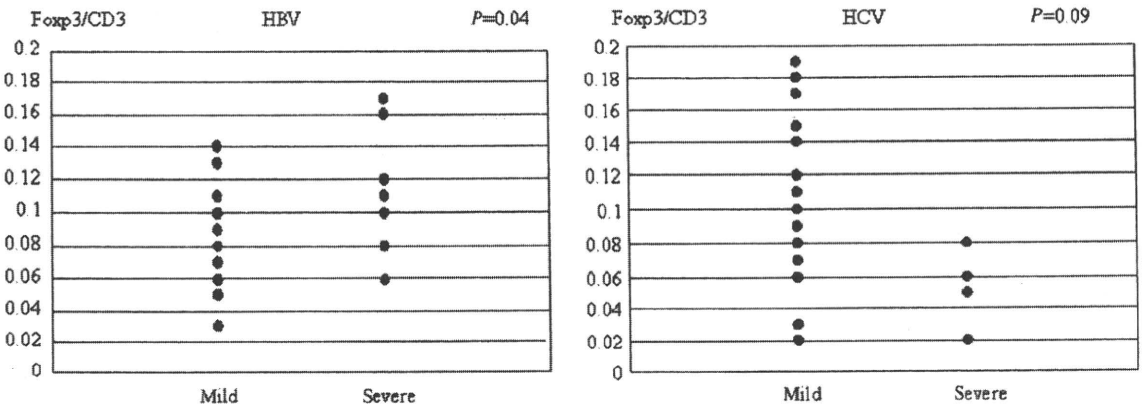


Fig. 4. Comparison between the ratio of Foxp3$^+$ Tregs in CD3$^+$ T cells and the histological fibrosis grade in chronic hepatitis B and in chronic hepatitis C. A significant difference was observed in chronic hepatitis B using the Mann–Whitney *U* test ($P = 0.04$).

In this study, the transaminase level was > 1000 IU/ml in some of the HBV cases, but in all HCV cases, it was < 300 IU/ml. These results suggest that Treg could constantly suppress the flare of inflammation induced by HCV.

The prevalence of regulatory T cells in the peripheral blood of patients with autoimmune encephalitis or systemic lupus erythematosus (24) is higher in males than in females. Similarly, in the present study, the ratio of Foxp3$^+$ Tregs in CD3$^+$ T cells in HCV cases in the liver was significantly higher in females than in males. Sex hormones may act directly on Tregs to alter their functions because immune cells express oestrogen and androgen receptors (25, 26). In the HCV cases, most of the female patients were over 50 years old (76%) and in a postmenopausal state. These findings suggested that oestrogen deficiency is therefore associated with the Tregs in the liver.

Viral genotype, viral load and gender have been shown to be key factors associated with HCV clearance by interferon therapy (27, 28, 29). The mechanism responsible for this difference in interferon treatment outcome is unknown. In this study, in HCV cases, the variables that were significantly associated with the ratio of Foxp3$^+$ Tregs in CD3$^+$ T cell were genotype2 and male gender. These results suggested that regulatory T cells in the liver may be related to the clearance of HCV by interferon therapy and may be a useful indicator for interferon therapy. The ratio of Foxp3$^+$ Tregs in CD3$^+$ T cells showed a significant inverse correlation with the HCV viral load. Tregs were induced by antigen-presenting cells such as dendritic cells (30). Therefore, a decreasing number of Tregs may indicate an impairment of antigen-presenting cells; conversely, the presence of Tregs may represent a normal function of antigen-presenting cells. Taken together, it is possible that, in CHB, regulatory T cells suppress the excessive activity of effector T cells during acute exacerbation, and that, in CHC, the presence of regulatory T cells may be a marker of the normal response of the host immunosystem against HCV.

References

1. Chisari FV, Ferrari C. Hepatitis B virus immunopathology. *Springer Semin Immunopathol* 1995; 17: 261–8.
2. European Association for the Study of the Liver. International consensus conference on hepatitis C. Paris, 26–28 February 1999. *J Hepatol* 1999; **30**: 956–61.
3. Sakaguchi S. Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 2004; **22**: 531–62.
4. Piccirillo CA, Thornton AM. Cornerstone of peripheral tolerance: naturally occurring CD4+ CD25+ regulatory T cells. *Trends Immunol* 2004; **25**: 374–80.
5. Jiang H, Chess L. An integrated view of suppressor T cell subsets in immunoregulation. *J Clin Invest* 2004; **114**: 1198–20.
6. Sakaguchi S, Sakaguchi N, Shimizu J, *et al.* Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol Rev* 2001; **182**: 18–32.
7. Maloy KJ, Powrie F. Regulatory T cells in the control of immune pathology. *Nat Immunol* 2001; **2**: 816–22.
8. Hori S, Sakaguchi S. Foxp3: a critical regulator of the development and function of regulatory T cells. *Microbes Infect* 2004; **6**: 745–51.
9. Coutinho A, Hori S, Carvalho T, *et al.* Regulatory T cells: the physiology of autoreactivity in dominant tolerance and "quality control" of immune responses. *Immunol Rev* 2001; **182**: 89–98.
10. Lan RY, Ansari AA, Lian ZX, *et al.* Regulatory T cells: development, function and role in autoimmunity. *Autoimmun Rev* 2005; **4**: 351–63.
11. Furuno K, Yuge T, Kusuhara K, *et al.* CD25+ CD4+ regulatory T cells in patients with Kawasaki disease. *J Pediatr* 2004; **145**: 385–90.
12. Kukreja A, Cost G, Marker J, *et al.* Multiple immuno-regulatory defects in type-1 diabetes. *J Clin Invest* 2002; **109**: 131–40.
13. Shevach EM, Piccirillo CA, Thornton AM, *et al.* Control of T cell activation by CD4+ CD25+ suppressor T cells. *Novartis Found Symp* 2003; **252**: 24–36; discussion 36–44, 106–114.
14. Kim JD, Choi BK, Bae JS, *et al.* Cloning and characterization of GITR ligand. *Genes Immun* 2003; **4**: 564–9.
15. Sakaguchi S. Naturally arising Foxp3-expressing CD25+ CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* 2005; **6**: 345–52.
16. Fehervari Z, Sakaguchi S. CD4+ Tregs and immune control. *J Clin Invest* 2004; **114**: 1209–17.
17. Gondek DC, Lu LF, Quezada SA, *et al.* Cutting edge: contact-mediated suppression by CD4+ CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol* 2005; **174**: 1783–6.
18. Andersson J, Boasso AC, Nilssonc J, *et al.* The prevalence of regulatory T cells in lymphoid tissue is correlated with viral load in HIV-infected patients. *J Immunol* 2005; **174**: 3143–7.