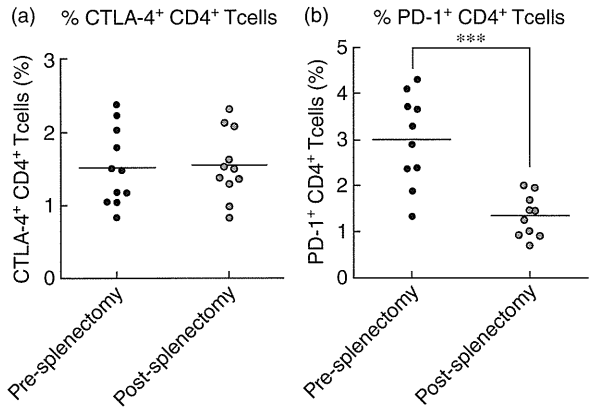

Fig. 7. Expression of programmed death-1 (PD-1) but not cytotoxic T lymphocyte associated antigen-4 (CTLA-4) on $CD4^+$ T cells change after splenectomy. CTLA-4 and PD-1 expression on $CD4^+$ T cells were analysed before and after splenectomy. There were no remarkable changes in the CTLA-4^+ (a), but the frequencies of PD-1^+ $CD4^+$ T cells decreased significantly after splenectomy (b) (***$P < 0{\cdot}001$).

stimulation [17,23]. Exhaustion of HCV-specific T cells [24,25], increased frequency of T_{regs} [26,27] and cytokine production patterns are critical host factors affecting the outcome of disease [28,29]. DC functions in HCV infection remain controversial, although most studies describe either reduced expression levels of co-stimulatory molecules [30] or a decreased allostimulatory capacity of DC [31]. Very little is known about the role of splenic T cells in HCV-related disease. The present study was therefore aimed to clarify the role of splenic $CD4^+$ T cells in patients with HCV related LC. To this end we took advantage of a cohort of patients who underwent splenectomy to treat severe thrombocytopenia in HCV-related LC prior to IFN-α therapy. This report demonstrates for the first time that markers associated traditionally with peripheral tolerance are also promoted by the spleen in HCV-related disease. Indeed, patients with HCV-related LC demonstrate splenic CD4 inhibitory signalling have higher levels of splenic CD4 T_{regs}, and of PD-L1- and PD-L2-expressing cells. Moreover, blocking of PD-1/PD-1 ligand interaction reconstitutes proliferative and cytokine responses.

Importantly, splenectomy is followed by an increasing ratio of IFN-γ to IL-10 and a reduction of PD-1-expressing $CD4^+$ T cells in peripheral blood.

One of the major mechanisms involved in the induction of peripheral tolerance is thought to involve the function of T_{regs} [32,33] by their interaction with APCs, including the generation of co-stimulation signals and a role for the programmed cell death pathway (PD-1/PD-1 ligands) [22,34]. Up-regulation of PD-1 on $CD4^+$ and $CD8^+$ T cells has been reported in the livers of HCV hepatitis patients [28]. A significant up-regulation of PD-1 expression was detected on exhausted virus-specific T cells in a mouse model with chronic lymphocytic choriomeningitis virus infection, and promoted viral persistence [20]. Our data demonstrate that this pathway is also involved in T cell signalling among splenic cells of patients with HCV-related LC. Subjects described herein have reduced T cell proliferation and lower levels of IFN-γ production upon CD3 stimulation of $CD4^+$ T cells, and this is associated with high frequencies of CTLA-4^+ or PD-1^+ T cells, but no correlation was noted with the frequency of $CD28^+$ or $CD154^+$ T cells. This result correlates with the up-regulation of PD-L1 and PD-L2 in SMC. Blocking of PD-1 signalling on exhausted T cells results in the restoration of T cell function, with an increased proliferation, cytotoxicity and cytokine production [18,34].

Blockage of the programmed cell death pathway has also been suggested as a potential immune therapy to enhance the effector T cell responses during persistent HCV infection in a murine HCV model [35]. The inhibitory molecules PD-L1 and PD-L2 play a prominent role in suppressing activated T cells, via a cell contact-mediated mechanism [36]. In addition, these PD-L1/2-mediated suppressive functions are considered to be due to the suppression of IL-2 production [36]. PD-L1/2 has been identified by their distinct and different expression patterns.

Thus, whereas PD-L1 expression is noted primarily in haematopoietic and parenchymal cells [21], PD-L2 expression is restricted mainly to DC and macrophages [21]. Moreover, recent studies have also identified PD-L2 expression in endothelial cells which mediate immune tolerance [37,38]. The findings of functional studies of the differentiation between PD-L1 and PD-L2 still remain controversial, but indicate that PD-L1 and PD-L2 exert overlapping effects on T cell responses [22,38].

The immunohistochemistry findings in the current study reflect high PD-1 expression on spleen from patients with HCV-related LC. Interestingly, PD-1 ligand expression, especially PD-L2, is up-regulated in the spleen with HCV-related LC. The expression of PD-L1 and PD-L2 in SMC was high in comparison to expression in PBMC. Therefore, PD-L1 and PD-L2 expression by splenic cells may contribute to T cell responses, suggested by the recovery of the proliferation and IFN-γ production of SMC by blocking PD-1/PD-1 ligands. The spleen is a reservoir of PD-L1 and PD-L2 in patients with HCV-related LC; splenectomy may induce the recovery of IFN-γ production and peripheral $CD4^+$ T cell proliferation. Therefore, splenectomy in patients with HCV-related LC may not alter the generation of adaptive immune response of $CD4^+$ T cells which thus allows them to achieve a virological response with IFN therapy. Our data reveal more details of the immunobiology of HCV-specific immune response in the spleen. Clearly, more work is needed before a clinical recommendation.

Importantly, our study demonstrates that peripheral tolerance in patients with HCV-related LC is at least partially promoted by the splenic up-regulation of PD-1 ligands.

Disclosure

The authors have no conflicts of interest to declare.

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Improvement of Long-Term Outcomes in Hepatitis C Virus Antibody–Positive Patients with Hepatocellular Carcinoma after Hepatectomy in the Modern Era

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Abstract
Background The present study was conducted to clarify the causes of recent improvement of outcomes after hepatectomy in patients with hepatitis C (HC)–related hepatocellular carcinoma (HCC).
Methods From 1990 to 2006, 323 curative liver resections for HC-HCC were performed in our department. The patients were divided into two groups: early period (1990–1999: $n = 221$) and the late period (2000–2006: $n = 102$). Prognostic factors were determined to clarify the cause of the survival improvement in the modern era.
Results The overall survival rates for the patients in the early and late periods were 54.9 and 70.3% at 5 years, respectively ($P = 0.0005$). There was no difference in the recurrence-free survival rates between the two groups, although both survival without recurrence ($P = 0.0003$) and survival after recurrence ($P = 0.0063$) were significantly better in the late period than in the early period. Patients with better liver function, patients with interferon (IFN) therapy and patients with subsegmentectomy were selected more frequently, and the incidence of blood transfusion was decreased in the late period below the level recorded in the early period. For recurrent HCC, lipiodolization decreased and local ablation therapy increased in the late period. The independent prognostic factors for overall survival were preoperative serum levels of albumin and alanine aminotransferase, histological liver cirrhosis, tumor size, intrahepatic metastasis, histological grade, blood transfusion, and IFN therapy.
Conclusions In HC-HCC, survival was improved in the late period of the present study. Selection of patients with good liver function, no blood transfusion with reduction of blood loss, anti-hepatitis C virus therapy with IFN, and introduction of local ablation therapy for HCC recurrence may be related to the improved survival.

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Abbreviations

HCC	Hepatocellular carcinoma
HC	Hepatitis C
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
ICGR15	Indocyanine green retention test at 15 min
NLC	No liver cirrhosis
LC	Liver cirrhosis
AFP	Alpha-fetoprotein
DCP	Des-gamma-carboxy prothrombin
IFN	Interferon
SVR	Sustained viral responder
NR	Non-responder
LPD	Lipiodolization
RCTs	Randomized controlled trials

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the world [1]. The cause of HCC is infection with hepatitis viruses such as hepatitis B virus (HBV) and hepatitis C virus (HCV) [2]. The frequency of HCC originating from HCV infection is much higher than that of HCC originating from HBV infection.

The mechanism of HCV carcinogenesis is still unclear. Hepatitis C virus is an RNA virus that does not integrate

into the DNA of hepatocytes. Theoretically, HCV does not have a direct oncogenic mechanism; this is not the case for HBV [3].

We extensively examined the recurrence pattern of HCC and found that recurrence-free survival after hepatic resection for HCC is deeply related to the hepatitis activity in patients with HCV [4–6]. We also found that low concentrations of serum HCV RNA in HC-HCC are frequently present in 10-year survivors after hepatectomy [7]. These data suggest that the high frequency of recurrence owing to metachronous carcinogenesis in hepatitis C (HC)-HCC patients is caused by high inflammatory activity. Sasaki et al. [8] demonstrated that the potential for recurrence in later follow-up years is stronger for HC-HCC than for hepatitis B (HB)-HCC.

Regarding the control of HCV-based hepatitis activity, the beneficial effects of interferon (IFN) therapy after hepatectomy have been examined. However, the effects of IFN therapy on overall survival and recurrence-free survival in HC-HCC patients are still controversial.

In previous studies, the five-year survival rates after hepatectomy for HC-HCC were reported to be in the range of 50–60% [9–12]. Further improvement of the survival rates is reported here.

The present study was conducted to clarify the causes of the improvement in survival. The analyses initially focused on whether the overall improvement was based on better rates of recurrence-free survival or survival after recurrence. Further analyses focused on the effects of IFN therapy and therapeutic strategies for recurrent tumors.

Patients and methods

Patients

Anti-HCV antibodies (HCV-Ab) started to be measured in our institute in January 1990. From January 1990 to December 2006, 492 curative liver resections for HCC were performed at the Second Department of Surgery, Kyushu University. Curative resection was defined as complete macroscopic removal of the tumor. Among 492 patients, 74 (15%) were seropositive for hepatitis B surface antigen (HBs-Ag) and seronegative for HCV-Ab, 17 (3%) were seropositive for both HBs-Ag and HCV-Ab, 323 (66%) were seropositive for HCV-Ab and seronegative for HBs-Ag, and 78 (16%) were seronegative for both HBs-Ag and HCV-Ab. The HCC patients with positive HCV-Ab and negative HBs-Ag were regarded as HC-HCC and enrolled in the study. Written informed consent was obtained from all patients in this study.

Methods

Comparisons between the early and late study periods

The patients were divided into two groups: the early period (January 1990–December 1999; n = 221) and the late period (January 2000–December 2006; n = 102). The clinicopathological backgrounds and rates of overall survival, recurrence-free survival, and survival after recurrence were compared between the two groups to clarify the reasons why the outcomes improved in the late period. Thereafter, prognostic factors for overall survival, recurrence-free survival, and survival after recurrence were examined. In particular, anti-HCV therapy and therapies for recurrence were compared between the two groups.

Prognostic factors were determined to clarify the causes of the outcome improvement in the modern era. The prognostic factors were examined with respect to overall survival on the basis of the following variables: age ($\geq$60 years versus <60 years); gender (male versus female); serum albumin level (>3.8 g/dl versus $\leq$3.8 g/dl); total bilirubin level (>0.8 mg/dl versus $\leq$0.8 mg/dl); serum aspartate aminotransferase (AST) level (>40 IU/l versus $\leq$40 IU/l); serum alanine aminotransferase (ALT) level (>40 IU/l versus $\leq$40 IU/l); indocyanine green retention test at 15 min ($ICGR_{15}$) (>15.7% versus $\leq$15.7%); platelet number (>124,000 /mm^3 versus $\leq$124,000 /mm^3); Child classification (A versus B or C); histological liver cirrhosis (no liver cirrhosis [NLC] versus liver cirrhosis [LC]); hepatitis activity (none versus mild versus severe); tumor size (>3.2 cm versus $\leq$3.2 cm); serum alpha-fetoprotein (AFP) level (>24 ng/ml versus $\leq$24 ng/ml); des-γ-carboxy prothrombin (DCP) level (>300 mAU/ml versus $\leq$300 mAU/ml); tumor differentiation (well differentiated or moderately differentiated versus poorly differentiated); microvascular invasion (absence versus presence); intrahepatic metastasis (absence versus presence); TNM stage according to the Liver Cancer Study Group in Japan [13] (I, II, or III versus IV); operative procedures (partial hepatectomy, subsegmentectomy, segmentectomy, or bisegmentectomy versus trisegmentectomy); surgical margin (>0 mm versus 0 mm); operative time (>300 min versus $\leq$300 min); estimated blood loss (>1,000 ml versus $\leq$1,000 ml); blood transfusion (absence versus presence); postoperative complications (absence versus presence); past IFN therapy before hepatectomy (sustained viral responder [SVR] or non-responder [NR] versus no therapy); postoperative IFN therapy (SVR or NR versus no therapy); past or postoperative IFN therapy (SVR or NR versus no therapy). Hepatitis activity was defined by the METAVIR scoring system [14]. All samples were evaluated in a blinded fashion by an independent experienced liver pathologist on the basis of the necroinflammatory activity (A). In this

study the original scores were defined as follows: A0 and A1, mild activity; A2, moderate activity; A3, severe activity. The measurement of serum DCP was as described previously [15].

The clinicopathological backgrounds of the patients with tumor recurrence were compared between the early and late study periods. Prognostic factors for overall survival and survival after recurrence in the patients with HCC recurrence were examined by univariate and multivariate analyses.

Therapies for recurrent HCC

We performed lipiodolization (LPD), repeat hepatectomy, local ablation therapy, and other therapies for recurrent HCC. Lipiodolization is a selective regional cancer chemotherapy that employs Lipiodol plus an anticancer drug; it has been reported elsewhere [16]. Local ablation therapy included percutaneous ethanol injection therapy, microwave coagulation therapy, and radiofrequency ablation therapy. Other therapies included systemic chemotherapy with 5-fluorouracil (5-FU) and hepatic arterial infusion therapy with cisplatin (CDDP) and 5-FU [17]. Our strategy for recurrent HCC in the early period was as follows. Re-resection was the first choice [18]. If re-resection could not be performed, LPD was the second treatment choice. In the late period, re-resection was also the first choice when the recurrent HCC was >2 cm in diameter, the number of tumors was small (≤3), and the liver function was preserved. Local ablation therapy was considered when the recurrent HCC was small (≤2 cm) and the number of tumors was small (≤3). Lipiodolization was mainly chosen for multiple (≥3) recurrences.

Histological study

All of the resected specimens were cut into serial 5–10 mm thick slices and fixed in 10% formalin. After macroscopic examination, the slice with the greatest dimensions was trimmed for embedding in paraffin and cut into 4 μm microscopic sections. The sections were stained with hematoxylin and eosin. Tumor differentiation, microvascular invasion, intrahepatic metastasis, and histological liver cirrhosis were examined.

IFN therapy

The type, dosage, and duration of IFN therapy varied. The response to IFN therapy was determined virologically and biochemically. Sustained viral response after IFN therapy was defined as a return of the ALT activity to within the reference range and no detectable serum HCV RNA for at least six months after the end of the IFN therapy. All other responses were defined as NR. The serum samples were tested for HCV-Ab with an enzyme-linked immunosorbent assay (OrthoDiagnostic Systems, Tokyo, Japan). Serum HCV RNA was assayed by a reverse transcriptase-nested polymerase chain reaction with primers derived from a conserved 5′-untranslated region of the viral genome, and by a branched DNA probe method (Quantiplex HCV-RNA; Chiron Corp., Emeryville, CA).

The impacts of IFN therapy in the levels of serum albumin at the times of the first hepatectomy and recurrence were examined to clarify the effects of IFN therapy.

Follow-up strategy and recurrence pattern

After discharge, all patients were examined for recurrence by ultrasonography and tumor markers such as AFP and DCP every month, and by CT every six months. When recurrence was suspected, additional examinations such as hepatic angiography were performed.

Statistical analysis

All data are expressed as means ± standard error. Independent χ^2 tests were used for categorical variables. Continuous variables were compared by unpaired *t*-tests. The serum albumin levels at the times of the first hepatectomy and recurrence were assessed by paired *t*-tests. The survival curves were analyzed by the Kaplan–Meier method and compared with the log-rank test. A Cox proportional hazards model was used for multivariate analysis. Only those variables showing a statistically significant ($P \leq 0.05$) relationship with survival in univariate analyses were included in the overall multivariate Cox model. StatView software (version 4.11; Abacus Concepts Inc., Berkeley, CA) running on a Macintosh computer was used for the adjustment of all covariates and stepwise regression analyses. Values of $P < 0.05$ were considered statistically significant.

Results

The overall survival rates of the patients in the early and late study periods are shown in Fig. 1. The overall survival rates in the early and late periods were 71.0 and 82.6% at 3 years, and 54.9 and 70.3% at five years, respectively, and this difference was significant ($P = 0.0005$). The mean survival periods of the patients in the early and late periods were 6.0 ± 4.4 years and 4.2 ± 2.5 years, respectively ($P = 0.0001$). In the course of the study, 195 of the patients enrolled in this study died. An examination of the causes of death revealed that 161 patients (83%) died of cancer, 28 patients (14%) died of liver failure, and six

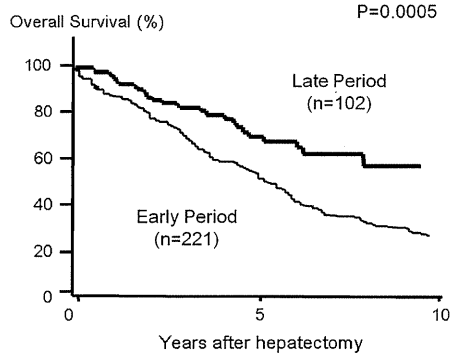

Fig. 1 Comparison of the overall survival rates between the early and late study periods. The overall survival rates in the early and late periods were 71.0 and 82.6% at 3 years, and 54.9 and 70.3% at 5 years, respectively, and this difference was significant ($P = 0.0005$)

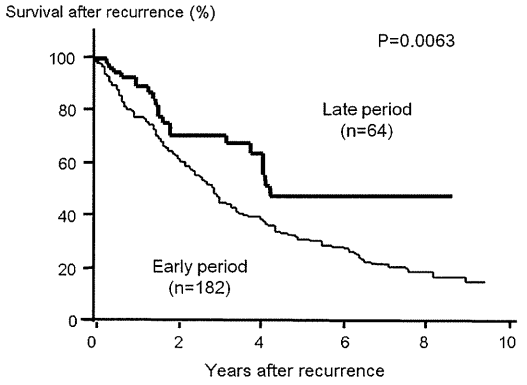

Fig. 3 Comparison of the survival rates after recurrence between the early and late study periods. The difference between the two groups is significant ($P = 0.0063$)

patients (3%) died of other miscellaneous causes, such as sepsis. In the early study period, 140 patients (83%) died of cancer, 22 patients (13%) died of liver failure, and six patients (4%) died of other causes. In the late period, 21 patients (78%) died of cancer and 6 (21%) died of liver failure. There was no significant difference in the causes of death between the early and late periods ($P = 0.3011$).

Furthermore, the overall survival rates of the patients with and without HCC recurrence were examined in the early and late periods. The five year survival rates without recurrence in the early and late periods were 56.7 and 82.4% ($P = 0.0002$), respectively. The five year survival rates with recurrence in the early and late periods were 47.3 and 65.9% ($P = 0.033$), respectively.

The recurrence-free survival rates of the patients in the early and late periods are shown in Fig. 2. There was no significant difference between the two groups. The survival rates after recurrence of the patients in the early and late periods are shown in Fig. 3. The survival after recurrence was significantly better in the late period than in the early period ($P = 0.0063$).

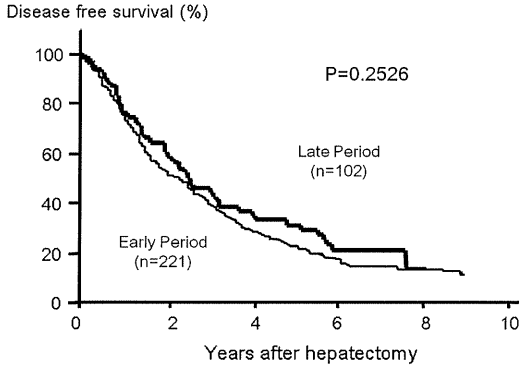

Fig. 2 Comparison of the recurrence-free survival rates between the early and late study periods. There is no significant difference between the two groups

The clinicopathological backgrounds of the patients in the early and late periods are shown in Table 1. The patients in the late period were significantly older than those in the early period. Regarding liver function, the serum albumin levels were significantly higher in the late period than in the early period, whereas the $ICGR_{15}$ values were significantly lower in the late period than in the early period. Thus, patients with good liver function were selected as having this indication for hepatectomy in the late period. The preoperative DCP values were significantly higher in the late period than in the early period. Regarding the operative procedures, subsegmentectomy increased and bisegmentectomy decreased in the late period. Consequently, the resected liver volume was significantly lower in the late period than in the early period. Estimated blood loss, incidence of blood transfusion, and postoperative complications were significantly decreased in the late period. The incidence of IFN therapy was more frequent in the late period than in the early period.

The prognostic factors for overall survival by univariate analyses are shown in Table 2. These factors were serum albumin level, AST level, platelet number, Child classification, histological liver cirrhosis, tumor size, AFP level, DCP level, tumor differentiation, microvascular invasion, intrahepatic metastasis, TNM stage, estimated blood loss, blood transfusion, postoperative complications, past IFN therapy before hepatectomy, postoperative IFN therapy, and past or postoperative IFN therapy.

The prognostic factors for overall survival by multivariate analyses are shown in Table 3. The significant prognostic factors were serum albumin level, AST level, histological liver cirrhosis, tumor size, intrahepatic metastasis, histological differentiation, blood transfusion, and past or postoperative IFN therapy.

Table 1 Comparisons of the clinicopathological factors between the early and late study periods

Backgrounds	Early period ($n = 221$)	Late period ($n = 102$)	P Value
Age	62 ± 0.5	68 ± 0.7	0.0001
Sex (male/female)	169/52	74/28	0.4913
Serum albumin, g/dl	3.8 ± 0.02	3.9 ± 0.04	0.0001
Total bilirubin, mg/dl	0.8 ± 0.02	0.9 ± 0.03	0.4197
$ICGR_{15}$, (%)	19.2 ± 0.7	16.7 ± 0.8	0.0213
Platelet count, $10^4/mm^3$	12.6 ± 4.4	13.3 ± 5.7	0.3585
Child classification (A:B+C)	168:53	84:18	0.3164
Histological hepatitis grade (None: mild: severe)	19:184:18	9:91:2	0.0961
Histological liver fibrosis (cirrhosis)	106 (48%)	38 (37%)	0.0716
Tumor size, cm	3.6 ± 0.2	3.6 ± 0.3	0.8714
AFP, ng/ml	1,665 ± 609	4,976 ± 4,029	0.2513
DCP, mAU/ml	889 ± 375	3,372 ± 1,145	0.0089
Tumor differentiation (poor)	67 (30%)	24 (24%)	0.1464
Mvi	81 (37%)	36 (35%)	0.8047
Im	45 (20%)	29 (28%)	0.1184
Stage (I:II:III:IVA)	31:91:85:14	24:41:30:7	0.1585
Operative procedures			0.0522
Partial resection	85 (39%)	38 (37%)	
Subsegmentectomy	35 (16%)	29 (28%)	
Segmentectomy	36 (16%)	16 (16%)	
Bisegmentectomy	61 (28%)	16 (16%)	
Trisegmentectomy	4 (2%)	3 (3%)	
Operative time, min	313 ± 7	326 ± 10	0.0641
Resected liver volume, g	313 ± 28	220 ± 28	0.0381
Estimated blood loss, g	1,702 ± 95	740 ± 62	0.0001
Blood transfusion (+)	82 (37%)	14 (14%)	0.0001
Postoperative complication	135 (61%)	49 (48%)	0.0301
Postoperative mortality	5 (2.2%)	0	0.3308
IFN therapy (yes)[a]	20 (9%)	37 (36%)	0.0001

$ICGR_{15}$ indocyanine green retention test at 15 min, *AFP* α-fetoprotein, *DCP* des-γ-carboxy prothrombin, *Mvi* microvascular invasion, *Im* intrahepatic metastasis, *Stage* TNM stage, defined by the Liver Cancer Study Group of Japan, *IFN* therapy patients who underwent past or postoperative interferon therapy for hepatitis C–derived hepatitis

The clinicopathological backgrounds of the patients with tumor recurrence in the early and late periods are shown in Table 4. At the time of the first hepatectomy, the serum albumin levels were significantly higher in the late period than in the early period. At the time of recurrence, the serum albumin levels were significantly higher in the late period than in the early period. The recurrent tumor sizes were significantly smaller in the late period than in the early period. Past or postoperative IFN therapy was more frequent in the late period than in the early period. The therapies for recurrent HCC were compared between the two groups. Lipiodolization was chosen more frequently in the early period than in the late period. Instead of LPD, the incidence of local ablation therapy increased in the late period.

The prognostic factors relating to survival after recurrence by univariate analyses are shown in Table 5. At the time of the first hepatectomy, serum albumin level, intrahepatic metastasis, microvascular invasion, tumor differentiation, and TNM stage were significant prognostic factors. Past or postoperative IFN therapy was also a significant prognostic factor. At the time of recurrence, serum albumin level, recurrent tumor size, and recurrence pattern were significant prognostic factors.

The prognostic factors relating to survival after recurrence by multivariate analyses are shown in Table 6. The significant prognostic factors were intrahepatic metastasis, past or postoperative IFN therapy, and therapy for recurrence.

The survival rates based on therapies for recurrence are shown in Fig. 4. There was no significant difference in survival after recurrence between re-resection and local ablation therapy, and both types of therapy were significantly superior to other therapies. The serum albumin levels were significantly higher in the re-resection group than in the local ablation therapy group, and the tumor sizes were significantly larger in the re-resection group than in the local ablation therapy group (Table 7).

Table 2 Univariate analyses for prognostic factors related to overall survival in 323 patients who underwent curative hepatic resections for HCV-related HCC

Factors	Survival rates				*P* Value
	1 year	3 year	5 year	7 year	
Age, years					0.0786
<60 ($n = 166$)	91.0	76.4	61.0	44.7	
≥60 ($n = 157$)	89.7	72.7	56.3	38.6	
Sex					0.7781
Male ($n = 243$)	89.7	72.7	56.3	38.6	
Female ($n = 80$)	91.0	76.0	59.8	40.9	
Albumin, g/dl					0.0001
≤3.8 ($n = 174$)	86.8	70.4	52.0	33.5	
>3.8 ($n = 149$)	94.6	79.4	68.3	53.0	
Total bilirubin, mg/dl					0.4934
≤0.8 ($n = 188$)	91.5	73.4	61.2	43.4	
>0.8 ($n = 135$)	88.7	76.2	56.1	39.6	
AST, IU/l					0.0098
≤40 ($n = 74$)	94.4	78.0	65.0	56.7	
>4 0 ($n = 249$)	89.1	73.6	57.2	37.7	
ALT, IU/l					0.5584
≤40 ($n = 72$)	94.3	70.5	61.0	47.1	
>40 ($n = 251$)	89.2	75.5	58.8	37.7	
$ICGR_{15}$, %					0.1242
≤15.7 ($n = 141$)	89.1	72.8	63.2	46.4	
>15.7 ($n = 182$)	94.0	75.7	56.0	38.1	
Platelet count, 10,000/mm^3					0.0146
<12.4 ($n = 174$)	89.0	74.4	55.3	36.1	
≥12.4 ($n = 149$)	91.9	74.8	63.7	49.0	
Child classification					0.0072
A ($n = 251$)	90.8	76.5	61.1	45.1	
B+C ($n = 72$)	88.7	67.9	52.5	31.2	
Histological liver cirrhosis					0.0049
NLC ($n = 179$)	90.5	76.7	66.3	51.0	
LC ($n = 144$)	90.2	72.0	50.4	31.1	
Histological hepatitis grade					0.2585
Mild ($n = 28$)	78.6	64.3	48.9	39.1	
Moderate ($n = 275$)	90.9	75.8	60.2	44.3	
Severe ($n = 20$)	100	70.0	53.3	17.8	
Tumor size, cm					0.0001
≤3.2 ($n = 185$)	94.6	83.5	67.5	50.9	
>3.2 ($n = 138$)	84.9	63.1	47.7	30.8	
AFP, ng/ml					0.0041
<24 ($n = 162$)	93.2	81.3	80.6	49.2	
>24 ($n = 161$)	86.9	68.0	48.2	35.3	
DCP, mAU/ml					0.0067
<300 ($n = 227$)	93.4	77.9	61.6	45.7	
≥300 ($n = 96$)	83.8	65.9	53.5	32.6	
Tumor differentiation					0.0053
Well+moderate ($n = 232$)	92.4	80.0	63.3	46.3	
Poor ($n = 91$)	84.6	59.0	46.8	29.4	

Table 2 continued

Factors	Survival rates				*P* Value
	1 year	3 year	5 year	7 year	
Mvi					0.0200
Absence ($n = 207$)	93.2	79.3	65.2	47.2	
Presence ($n = 116$)	83.8	50.8	28.9	18.8	
Im					0.0001
Absence ($n = 250$)	80.3	63.6	51.3	41.0	
Presence ($n = 73$)	50.0	37.5	28.1	0	
Stage					0.0001
I ($n = 55$)	98.1	92.2	80.6	65.0	
II ($n = 133$)	91.7	78.0	61.6	42.1	
III ($n = 115$)	86.0	64.9	51.8	37.1	
IV ($n = 21$)	85.7	58.9	30.6	8.2	
Operative procedures					0.1620
Partial hepatectomy ($n = 123$)	92.7	76.2	57.8	40.7	
Subsegmentectomy ($n = 64$)	93.7	83.6	64.0	48.1	
Segmentectomy ($n = 52$)	98.1	82.0	71.1	49.3	
Bisegmentectomy ($n = 77$)	79.4	60.8	50.5	37.0	
Trisegmentectomy ($n = 7$)	85.7	57.1	42.9	14.3	
Surgical margin, mm					0.1112
0 ($n = 93$)	85.9	74.8	54.5	37.1	
>0 ($n = 230$)	92.2	74.2	61.0	44.0	
Operative time, min					0.8081
< 300 ($n = 165$)	91.5	74.9	61.7	40.6	
≥300 ($n = 158$)	89.0	73.2	55.4	43.3	
Estimated blood loss, ml					0.0002
<1,000 ($n = 156$)	94.1	81.5	66.9	53.2	
≥1,000 ($n = 167$)	86.8	67.2	52.1	33.6	
Blood transfusion					0.0001
Absence ($n = 228$)	92.1	79.5	67.2	51.2	
Presence ($n = 95$)	86.5	62.7	41.3	22.6	
Postoperative complication					0.0065
Absence ($n = 139$)	94.9	83.3	67.5	50.4	
Presence ($n = 184$)	86.9	67.7	52.8	35.8	
Past interferon therapy					0.0012
SVR ($n = 12$)	100	90.9	77.9	77.9	
NR ($n = 28$)	96.3	96.3	90.3	75.2	
No therapy ($n = 283$)	89.4	71.9	55.7	38.7	
Postoperative interferon therapy					0.0001
SVR ($n = 19$)	100	100	100	100	
NR ($n = 27$)	100	92.3	87.2	76.9	
No therapy ($n = 277$)	88.8	70.9	54.1	35.6	
Past or postoperative interferon therapy					0.0001
SVR ($n = 31$)	100	96.7	91.6	91.6	
NR ($n = 40$)	97.4	91.7	84.9	70.8	
No therapy ($n = 252$)	88.1	69.3	52.1	33.3	

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *NLC* non-liver cirrhosis, *LC* liver cirrhosis, *SVR* sustained viral responder, *NR* non-responder

Table 3 Multivariate analyses for prognostic variables of overall survival

Prognostic variables	Regression coefficient	Standard error	Hazard ratio	*P* Value
Albumin	2.729	0.165	1.570	0.0063
AST	2.272	0.203	1.587	0.0231
Histological LC	2.804	0.155	1.543	0.0050
Tumor size	2.958	0.156	1.585	0.0001
Im	3.824	0.161	1.848	0.0001
Histological grade				
Poor	2.254	0.159	1.431	0.0242
Blood transfusion	2.638	0.157	1.513	0.0083
Past or postoperative interferon therapy				
SVR	1.988	0.592	7.299	0.0008
NR	2.747	0.332	2.747	0.0023

AST aspartate aminotransferase, *LC* liver cirrhosis, *Im* intrahepatic metastasis, *SVR* sustained viral responder, *NR* non-responder

Table 4 Comparisons of the clinicopathological backgrounds of patients with tumor recurrence between the early and late study periods

Factors	Early period ($n = 182$)	Late period ($n = 64$)	*P* Value
At first hepatectomy			
Albumin (>3.8 g/dl)	86 (48%)	44 (69%)	0.0036
Child (A:B:C)	141:36:3	54:10:0	0.4144
$ICGR_{15}$ (>15.7%)	105 (58%)	36 (56%)	0.8832
Histological LC	84 (46%)	23 (36%)	0.1892
Histological grade (poor)	52 (28%)	15 (23%)	0.4167
Im (presence)	55 (30%)	13 (20%)	0.1900
Mvi (presence)	70 (38%)	22 (34%)	0.6527
AFP (> 24 ng/ml)	81 (46%)	30 (47%)	0.3078
DCP (>300 mAU/ml)	77 (42%)	35 (55%)	0.1438
At the time of recurrence			
Period to recurrence, days	1,033 ± 1,013	750 ± 611	0.0365
Albumin (>3.8 g/dl)	78 (43%)	43 (67%)	0.0028
Size (≥2 cm)	94 (52%)	22 (34%)	0.0152
Recurrent pattern			
Nodular: multiple: extrahepatic	126:39:16	43:15:6	0.9365
IFN therapy SVR:NR: no therapy	3:14:165	12:16:36	0.0001
Therapies for recurrence			0.0020
LPD	94 (52%)	21 (33%)	
Local ablation therapy	22 (12%)	14 (22%)	
Re-resection	46 (25%)	17 (27%)	
Others	1 (1%)	5 (8%)	
None	19 (10%)	7 (11%)	

ICGR15 indocyanine green retention test at 15 minutes, *LC* liver cirrhosis, *Im* intrahepatic metastasis, *Mvi* microscopic vascular invasion, *DCP* des-γ-carboxy prothrombin, *AFP* α-fetoprotein

The impacts of past or postoperative IFN therapy on the serum albumin levels at the times of the first hepatectomy and recurrence are shown in Table 8. In the complete response (CR) and nonresponse (NR) groups, the serum albumin levels did not decrease at the time of recurrence, although the serum albumin levels were significantly lower at the time of recurrence than at the time of the first hepatectomy in the no therapy group. The serum albumin levels at the time of recurrence were significantly higher in the SVR and NR groups than in the no therapy group. Interferon therapy was effective for preserving liver function in hepatectomized patients.

Discussion

A significant improvement of the long-term outcomes after hepatic resection in patients with HC-HCC was proven. Previous studies have reported low recurrence-free survival rates, such as 20–30% at five years after hepatic resection [9–12]. Similarly, we found that the overall recurrence-free survival rate at 5 years was 25.4%, and that the group recurrence-free rates at five years were 23.2% in the early period and 31.6% in the late period. There was no significant difference between the early and late periods. On the other hand, the survival rates after recurrence at 5 years were 23% in the early period and 42.8% in the late period, and this difference was significant. Another important cause of the improvement in outcomes in the late period seems to be improvement of the survival rates of the patients without HCC recurrence. The difference in the survival rates of the patients was statistically significant. Because the main cause of death in the patients without HCC recurrence was liver failure, this difference may be

Table 5 Univariate analyses of prognostic factors related to survival after recurrence in patients with hepatocellular carcinoma (HCC) recurrence

Factors	Survival rates				*P*-Value
	1 Year	3 Year	5 Year	7 Year	
At first hepatectomy					
Albumin					
(>3.8 g/dl; $n = 140$)	83.8	60.2	41.8	34.4	0.0066
(≤3.8 g/dl; $n = 115$)	78.7	44.9	26.5	16.1	
AST					
(<40 IU/l; $n = 125$)	83.1	53.7	35.1	29.1	0.3540
(≥40 IU/l; $n = 120$)	78.8	51.8	33.5	21.6	
$ICGR_{15}$					
(≤15.7%; $n = 104$)	80.3	52.7	34.5	28.0	0.7908
(>15.7%; $n = 141$)	82.1	53.6	34.1	23.0	
Histological LC					
(–; $n = 101$)	85.1	54.5	33.1	26.3	0.7089
(+; $n = 144$)	76.5	50.6	36.6	25.6	
Histological hepatitis grade					0.1816
Mild ($n = 21$)	80.0	68.0	32.0	14.0	
Moderate ($n = 204$)	77.6	54.1	32.0	15.3	
Severe ($n = 20$)	70.0	55.0	14.0	14.0	
Im					
(–; $n = 176$)	85.4	60.5	41.9	31.6	0.0001
(+; $n = 69$)	69.0	33.0	17.9	11.6	
Mvi					
(–; $n = 154$)	85.5	58.8	42.1	30.2	0.0099
(+; $n = 91$)	73.7	43.7	23.7	19.0	
Tumor differentiation					
Well + mode ($n = 201$)	85.4	58.0	40.0	29.6	0.0007
Poor ($n = 44$)	72.5	42.5	21.4	15.6	
Stage					
I ($n = 37$)	97.2	71.8	53.6	42.9	0.0127
II ($n = 115$)	82.9	51.1	33.1	24.0	
III ($n = 91$)	74.3	49.2	30.2	22.0	
IVA ($n = 2$)	50.0	0	0	0	
Past or postoperative interferon therapy					0.0001
SVR ($n = 15$)	85.7	75.0	75.0	75.0	
NR ($n = 30$)	100	82.9	68.5	61.6	
No therapy ($n = 200$)	78.0	47.7	28.1	18.4	
At the time of recurrence					
Albumin					
(>3.8 g/dl; $n = 109$)	91.3	61.4	44.4	37.5	0.0001
(≤3.8 g/dl; $n = 121$)	71.8	39.4	23.6	14.6	
Tumor size					
(<2 cm; n Albumin 110)	91.3	61.4	44.4	32.1	0.0215
(≥2 cm; n Albumin 116)	75.6	48.1	29.9	22.3	
Recurrence pattern					
Nodular ($n = 169$)	89.5	63.1	41.0	31.2	0.0001
Multiple ($n = 54$)	69.5	35.2	23.6	17.7	
Extrahepatic ($n = 22$)	42.9	21.8	14.5	0	

AST aspartate aminotransferase, *ICGR15* indocyanine green retention test at 15 minutes, *LC* liver cirrhosis, *Im* intrahepatic metastasis, *Mvi* microscopic vascular invasion, *Stage* TNM stage defined by the Liver Cancer Study Group of Japan, *SVR* sustained viral responder, *NR* non-responder

Table 6 Multivariate analyses for prognostic variables of survival after recurrence

Prognostic variables	Regression coefficient	Standard error	Hazard ratio	*P* Value
First hepatectomy				
Im	3.104	0.211	1.927	0.0019
Past or postoperative interferon (IFN) therapy				
SVR	1.809	0.211	7.353	0.0405
NR	3.097	0.415	3.623	0.0020
At the time of recurrence				
Therapy for recurrence				
LPD	2.754	0.627	5.618	0.0059
LAT	3.432	0.689	10.64	0.0006
Re-resection	3.606	0.652	10.53	0.0003

Im intrahepatic metastasis, *NR* non-responder, *SVR* sustained viral responder, *LPD* lipiodolization, *LAT* local ablation therapy

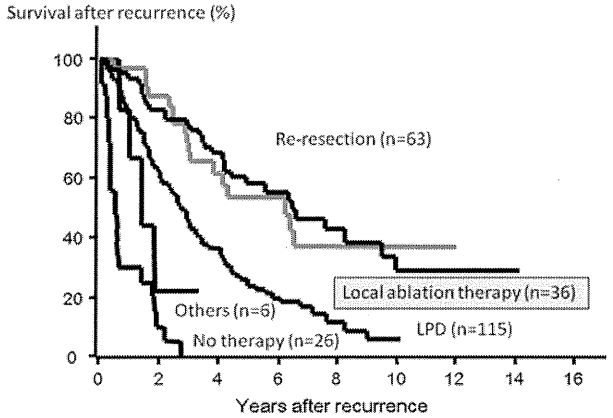

Fig. 4 Comparison of the survival rates after recurrence by therapies. The survival rates after re-resection, LPD and local ablation therapy are significantly better than those for no therapy and other therapies ($P = 0.0001$). The survival rates after re-resection ($P = 0.0001$) and local ablation therapy ($P = 0.0001$) are significantly better than that after lipiodolization. There is no significant difference between the patients who underwent re-resection and local ablation ($P = 0.7287$)

caused by the selection of patients with good liver function and increased rates of no blood transfusion with reduced blood loss in the late period. Therefore, the significant improvement in survival for both patients without recurrence and patients after recurrence seems to be the main cause of the improvement in the overall survival.

The causes of the improvement in the outcomes seem to be multifactorial. Previous studies regarding the improvement of outcomes after hepatic resection for HCC demonstrated that the following factors may influence the outcomes: (1) patient selection; (2) operative mortality; (3) recurrence-free survival; (4) postoperative surveillance; (5) management of HCC recurrence; and (6) IFN therapy. After multivariate analyses, the significant prognostic factors were liver function and hepatitis activity, as reflected by serum albumin and AST levels; histological liver cirrhosis; tumor invasiveness, as reflected by tumor size, intrahepatic metastasis, and histological grade; blood transfusion; and IFN therapy. Among these factors, there were no significant differences in recurrence-free survival

Table 7 Comparison of the clinical backgrounds of the patients in the treatments for recurrent HCC

Factors	Resection ($n = 63$)	LAT ($n = 36$)	LPD ($n = 115$)	No therapy ($n = 26$)	Others ($n = 6$)
Serum albumin	3.7 ± 0.1[a]	3.5 ± 0.1[a,b]	3.5 ± 0.1[a]	3.2 ± 0.1	3.6 ± 0.1
Total bilirubin	0.9 ± 0.1[c]	0.9 ± 0.1[c]	1.0 ± 0.1[c]	2.0 ± 0.1	0.9 ± 0.1
Rec. pattern,[c] I:II:III[d]	55:5:3	34:2:0	71:36:8	10:8:8	0:3:3
Tumor size, cm	2.4 ± 0.2[e]	1.7 ± 0.1	2.0 ± 0.1[f]	2.2 ± 0.3	1.5 ± 0.3

[a] $P < 0.05$ versus no therapy

[b] $P < 0.05$ versus resection

[c] $P < 0.05$ versus no therapy

[d] Recurrence pattern I is intrahepatic recurrence with no more than three nodules. Recurrence pattern II is intrahepatic recurrence with more than three nodules. Recurrence pattern III is distant metastasis

[e] $P < 0.05$ versus LAT

[f] $P < 0.05$ versus no therapy

Table 8 Impacts of interferon (IFN) therapy on the serum albumin levels at the times of the first hepatectomy and at recurrence

IFN response	Preoperative albumin, g/dl	Albumin at recurrence, g/dl	*P* Value
SVR ($n = 15$)	4.0 ± 0.3[a]	3.9 ± 07[a]	0.4455
NR ($n = 30$)	3.9 ± 0.4	3.8 ± 0.5[b]	0.0719
No therapy ($n = 200$)	3.8 ± 0.4	3.5 ± 0.5	0.0001

[a] $P < 0.01$, versus the serum albumin levels in patients with no IFN therapy

[b] $P < 0.01$, versus the serum albumin levels in patients with no IFN therapy

The *P* values were calculated for differences between the preoperative serum albumin levels and the serum albumin levels at the time of recurrence using paired *t*-tests

and postoperative surveillance. The incidence of operative complications was reduced and was a significant prognostic factor in univariate analyses, although it was not an independent prognostic factor after multivariate analysis.

Patients with good liver function were selected more frequently in the late period than in the early period. This may have been so because the patients with poor liver function in the late period were treated by local ablation therapy as a first therapeutic choice, and the incidence of local ablation therapy was more common in this period. Furthermore, patient selection on the basis of good liver function may have reduced the number of patients who died without HCC recurrence, mainly from liver failure.

Regarding the operative procedures, subsegmentectomy was selected over bisegmentectomy more frequently in the late period than in the early period. The reduction in the resected liver volume may be based on this change. There have been many reports suggesting a high frequency of recurrence owing to metachronous carcinogenesis in HC-HCC patients. Takano et al. [19] reported that the incidence of HCC was 2.7 times higher in chronic HC patients than in chronic HB patients. Because there was no clear evidence for the best operative procedure for HC-HCC patients in the early 1990s, hepatic lobectomy was our first choice to seek a cure for HCC in our institute [20]. Although Eguchi et al. [21] demonstrated that subsegmentectomy was superior to limited hepatic resection in terms of recurrence-free survival, not overall survival, using Japanese nationwide surveillance data, further examinations are necessary to determine whether this result is adequate for HC-HCC patients. Dahiya et al. [22] also reported a comparison of minor versus major hepatic resection for small HCC based on long-term follow-up data and concluded that the severity of cirrhosis and tumor characteristics affected long-term survival more than the type of resection in HCC. Major hepatic resection such as bisegmentectomy may be inadequate in HC-HCC patients, especially for small HCC, under the condition that a high incidence of metachronous carcinogenesis and preservation of liver function may be factors in long-term survival. Furthermore, we have shown that HCC patients with high DCP levels, large tumor sizes [15], and large portal venous perfusion defects on CT during arterioportography [23] frequently have microvascular invasion, and in this type of HCC, major hepatic resection would be recommended, whereas limited hepatic resection can be valid in HC-HCC [24, 25].

Previous reports have shown that hepatitis activity is an important factor for recurrence in HC-HCC. We have shown that the histological hepatitis activity and postoperative levels of transaminase are significant risk factors for HCC recurrence [4–6] in small HCC. These observations suggest that anti-HCV therapy with IFN after hepatectomy may reduce HCC recurrence and improve outcomes. Also in this study, anti-HCV therapy was one of the significant prognostic factors for outcome improvement after multivariate analyses. After excluding the patients who underwent IFN therapy, the difference in the outcomes between the early and late periods was still significant ($P = 0.0242$; data not shown) and the *P* value was much reduced. The survival rates after recurrence did not differ significantly between the two periods ($P = 0.0713$). Therefore, the cause of this outcome improvement was not solely based on IFN therapy. Nevertheless, IFN treatment seems to be one of the important factors in the improvement of the outcomes. The results of previous randomized controlled trials (RCTs) are controversial [26–29]. In all the trials, the beneficial effects for patients who achieved SVR after IFN therapy were clear. The results of the RCTs reported by Ikeda et al. [26] and Kubo et al. [27] showed that IFN therapy improved survival and/or recurrence-free survival. In contrast, the RCTs reported by Shiratori et al. [28] and Mazzaferro et al. [29] failed to show any beneficial effects on overall survival and recurrence-free survival. Nevertheless, they did show beneficial effects on late recurrence, and further follow-up may prove that there were beneficial effects on overall survival. The present study showed that SVR by IFN therapy clearly improved both recurrence-free survival and overall survival. Nonresponse failed to have any beneficial effects on recurrence-free survival but did have beneficial effects on overall survival. The serum

albumin level was preserved at the time of recurrence in patients who underwent IFN therapy, although the serum albumin levels were lower in patients who did not undergo IFN therapy. Furthermore, not only postoperative IFN therapy but also past IFN therapy had the same effects on the outcome after hepatectomy, as reported previously [30]. Kudo et al. [31] showed that low-dose intermittent IFN therapy improved the outcome in HC-HCC patients. If this regimen is applied to patients with NR, further improvement of the outcome may be achieved.

Blood transfusion is another independent prognostic factor [32–34], although estimated blood loss was not an independent prognostic factor. There have been several studies reporting adverse effects of blood transfusion at the time of surgery. The mechanism of the adverse effects of blood transfusion remains unclear. Matsumata et al. [32] reported that an association between transfusion and recurrence-free survival was only recognized in patients with intrahepatic metastasis-negative HCC. This may arise because a blood transfusion facilitates carcinogenesis of the liver.

For recurrent HCC, re-resection, local ablation therapy, LPD, other therapies and no therapy were performed. The survival rate after recurrence was significantly better in the late period than in the early period. Our data show that local ablation therapy was more frequently performed for recurrent HCC in the late period than in the early period. The incidence of LPD was reduced and the incidence of re-resection remained unchanged. In a comparison of LPD and local ablation therapy, the outcome of re-resection and local ablation therapy was superior to that of LPD in solitary recurrence, although there was no significant difference among the patients with other recurrence patterns (data not shown). In the early period, the first choice of treatment for solitary recurrence was hepatic resection. In cases with impaired liver function, LPD was performed. In the late period, local ablation therapy was selected for solitary recurrence with impaired liver function. This may be one of the causes of the improvement in survival after recurrence. There was no significant difference in the survival rates after recurrence between the re-resection group and the local ablation therapy group. Patients who underwent local ablation therapy had smaller recurrent tumors and worse liver function than those who underwent re-resection. In the late period, when the recurrent tumor was less than 2 cm in diameter and the number of nodules was no more than 3, local ablation therapy was considered to be the first choice of treatment. The clinical backgrounds between the two groups differed significantly, and therefor simple comparisons of their outcomes cannot be carried out. At the very least, local ablation therapy can be applied to small recurrent HCC after meticulous surveillance of the recurrent HCC. Taura et al. [10] showed beneficial effects of frequent local ablation therapy for intrahepatic recurrence on prolonged survival of patients with HCC undergoing hepatic resection.

No patients underwent liver transplantation for the first recurrence of HCC in this series. Liver transplantation was only performed in two patients after several treatments (data not shown), because of the deceased donor shortage in Japan. Because the long-term outcomes of liver transplantation are superior to those of other treatments in previous studies [35, 36], salvage transplantation for recurrent HCC should be considered. Cucchetti et al. [37] described the harm and benefits of primary liver resection and salvage transplantation for HCC. They demonstrated that the balance between harm for resected patients and benefits for the remaining waiting list patients depends on (a) the proportion of HCC candidates, (b) the percentage shifted to hepatic resection, and (c) the median expected time-to-transplant. The shortage of deceased donors is serious in Japan, and salvage transplantation should be considered in the very near future.

In conclusion, a significant improvement in the outcomes after hepatic resection was proven for patients with HC-HCC. In patients with HC-HCC, preservation of the liver function may be an important clue for long-term survival.

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Expression Profiles of Genes Associated With Viral Entry in HCV-Infected Human Liver

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Recent studies have demonstrated that several cellular factors are involved in entry of hepatitis C virus (HCV) into host cells. Detailed gene expression profiles of these factors in HCV-infected livers have not been reported for humans. Transcriptional levels of LDL receptor (LDLR), CD81, scavenger receptor class B type I (SR-BI), claudin-1, and occludin genes in liver samples from patients with chronic hepatitis C were investigated. Serum levels of LDL-cholesterol (LDL-C) and HCV core antigen were also evaluated, and expression of claudin-1 and occludin were immunohistochemically analyzed. Compared with normal liver, transcription of LDLR and claudin-1 genes was significantly suppressed ($P < 0.0001$) and occludin transcription was significantly up-regulated in HCV-infected livers ($P < 0.0001$). Significant positive correlations were found for LDLR versus occludin, LDLR versus claudin-1, occludin versus claudin-1, and CD81 versus SR-BI in HCV-infected ($P = 0.0012$, $P < 0.0001$, $P = 0.0004$, and $P < 0.0001$, respectively) and normal livers ($P < 0.0001$, $P = 0.0051$, $P < 0.0001$, and $P < 0.0001$, respectively). Positive correlation was observed between serum levels of HCV core antigen and LDL-C ($P = 0.0147$), with their levels negatively correlated to LDLR ($P = 0.0270$ and $P = 0.0021$, respectively). Immunohistochemically, hepatocellular expression of claudin-1 and occludin was increased in HCV-infected livers. Different levels of expression were demonstrated at the mRNA and protein levels for occludin and claudin-1 in HCV-infected and normal livers. Correlation of elements associated with viral entry was comparable in HCV-infected and normal livers. ***J. Med. Virol. 83:921–927, 2011.*** © 2011 Wiley-Liss, Inc.

KEY WORDS: occludin; claudin-1; LDL receptor; CD81; scavenger receptor class B type I

INTRODUCTION

The cellular factors or receptors required for hepatitis C virus (HCV) entry/infection have recently been reported and LDL receptor (LDLR), CD81, scavenger receptor class B type I (SR-BI), claudin-1, and occludin are considered to be essential molecules for HCV entry into hepatocytes [Owen et al., 2009; Perrault and Pécheur, 2009; Pietschmann, 2009; Tang and Grisé,

Abbreviations used: LDL-C, LDL-cholesterol; LDLR, LDL receptor; MTP, microsomal triglyceride transfer protein; RBBP6, retinoblastoma binding protein 6; RT-qPCR, quantitative real-time reverse transcription-polymerase chain reaction; SR-BI, scavenger receptor class B type I.

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Study design, sample collection, supervision of laboratory work, data analysis, manuscript writing, and administrative work were done by M. Nakamuta; T. Fujino, R. Yada, Y. Aoyagi, and K. Yasutake performed the laboratory work; M. Kohjima, K. Fukuizumi, T. Yoshimoto, and N. Harada did sample collection; M. Yada, M. Kato, and K. Kotoh did the supervision of laboratory work and data analysis; A. Taketomi, and Y. Maehara did sample collection; M. Nakashima critically reviewed the manuscript; M. Enjoji did study design, data analysis, manuscript writing, and administrative work.

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2009]. A current model predicts a multistep process for entry and infection. (i) The HCV particle consists of a nucleocapsid that is surrounded by a lipid bilayer in which the envelope formed by E1/E2 heterodimers is anchored to a host-cell-derived lipid membrane [Perrault and Pécheur, 2009]. The majority of HCV in the blood is found associated with β-lipoproteins. HCV particles associated with apolipoprotein E non-specifically attach to the cell surface molecules, LDLR, and glycosaminoglycans, an event that precedes virus interaction with CD81 and SR-BI [Burlone and Budkowska, 2009; Owen et al., 2009; Perrault and Pécheur, 2009]. (ii) The next step is specific binding of HCV envelope proteins to the entry receptors CD81 and SR-BI, which localize to the basolateral surface of polarized epithelial cells and interact with HCV envelope protein E2 [Nakagawa et al., 2004; Levy and Shoham, 2005]. CD81/E2 engagement triggers Rho family GTPase-dependent actin rearrangements, which allows lateral movement of the CD81/E2 complex to the tight-junctions, where the CD81/E2 complexes come into contact with claudin-1 and occludin [Brazzoli et al., 2008]. (iii) Claudin-1 and occludin, which are critical components of tight-junctions, have been identified as critical HCV hepatocyte entry factors at a late event, presumably after virus binding and interaction with CD81, and could be crucial factors for HCV internalization [Evans et al., 2007; Benedicto et al., 2009; Liu et al., 2009; Ploss et al., 2009]. No direct HCV–claudin-1 or HCV–occludin interaction has been demonstrated [Evans et al., 2007; Ploss et al., 2009; Krieger et al., 2010], but claudin-1 and occludin co-immunoprecipitate with both HCV envelope proteins, and also form complexes with CD81 and LDLR. Therefore, entry steps mediated by claudin-1 and occludin involve similar mechanisms that take place at the tight junction [Benedicto et al., 2008; Yang et al., 2008]. The tight-junction protein claudin-1 localizes to the basolateral surfaces of hepatocytes [Reynolds et al., 2008] and the non-junctional claudin-1 may be involved in HCV entry [Evans et al., 2007; Cukierman et al., 2009]. Claudin-1 associates with CD81, and this complex is essential for HCV infection [Harris et al., 2008; Harris et al., 2010]. (iv) The final step is presumably clathrin-dependent endocytosis, followed by transport of the viral particles to the endosome [Owen et al., 2009; Perrault and Pécheur, 2009; Pietschmann, 2009; Tang and Grisé, 2009].

Subsequently, non-HCV-permissive human and non-human cell lines become susceptible to HCV when CD81, SR-BI, claudin-1, and occludin are expressed, and CD81 and occludin function as human-specific HCV entry factors [Ploss et al., 2009]. However, the genes for these viral-entry-associated proteins in HCV-infected liver have not been thoroughly investigated in humans. In this study, the transcriptional levels of LDLR, CD81, SR-BI, claudin-1, and occludin genes were examined in liver tissue samples obtained from patients with chronic HCV infection, and their reciprocal relationships were estimated. The expression of claudin-1 and occludin proteins in liver tissue was immunohistochemically analyzed. The level of correlation for these viral entry-associated genes with serum concentrations of LDL-cholesterol (LDL-C) and HCV core antigen were also investigated. LDL-C levels may be related to HCV infection and possibly correlate with the outcomes of interferon treatment [Economou et al., 2008; Torres and Harrison, 2008; Sezaki et al., 2009]. This is the first report investigating the expression profiles of genes associated with HCV entry in HCV-infected human livers.

MATERIALS AND METHODS

Tissue samples were obtained by liver biopsy from 103 patients with chronic hepatitis C prior to any antiviral treatment. These patients were admitted to the Kyushu Medical Center and Kyushu University Hospital in 2007–2009. The background characteristics of these patients are shown in Table I. As a control, normal liver tissue was obtained from 35 living donors that had undergone liver transplantation and whose liver function tests and histological findings were completely normal. The study protocol was approved by the Ethics Committee of Kyushu Medical Center and Kyushu University Hospital, and written informed consent was obtained from all patients.

Blood samples for serum biochemistry were collected on the day of liver biopsy. Serum levels of HCV core antigen were measured using a chemiluminescent enzyme immunoassay (Lumipulse Ortho HCV Ag; Ortho Clinical Diagnostics, Tokyo, Japan).

Gene expression was examined by quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR) and compared between HCV-infected and normal livers. The PCR primers for the amplification of LDLR, CD81, SR-BI, claudin-1, occludin, and retinoblastoma-binding protein 6 (RBBP6) are listed in Table II. Total RNA was prepared from the tissue samples using TRIzol reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized from 1.0 μg RNA using a GeneAmp RNA PCR kit (Applied Biosystems, Branchburg, NJ) in conjunction with random hexamers. The RT-qPCR was performed with a LightCycler-FastStart DNA Master SYBR Green 1 kit (Roche, Basel, Switzerland) in accordance with the manufacturer's instructions. The reaction mixture (20 μl) contained LightCycler-FastStart DNA Master SYBR Green 1,

TABLE I. Characteristics of Patients Enrolled in this Study

Number	103
Sex (male/female)	40/63
Age (years)	56.06 ± 11.65 (23–74)
HCV genotype (1b/2a or 2b)	62/41
Grading (A0/A1/A2/A3)	0/50/52/1
Staging (F0/F1/F2/F3/F4)	5/48/25/24/1
Total cholesterol (mg/dl)	177.37 ± 32.96 (114–265)
Triglyceride (mg/dl)	94.32 ± 38.41 (34–185)
HDL-cholesterol (mg/dl)	54.15 ± 15.45 (25–106)
LDL-cholesterol (mg/dl)	104.14 ± 27.79 (45–167)
ALT (IU/l)	69.76 ± 79.84 (9–482)
HCV core antigen (fmol/l)	7214.37 ± 7724.04 (20–37500)

TABLE II. Sequences of Primers Used for RT-qPCR

Genes	Forward (5′3′)	Reverse (5′3′)
LDLR	CAACGGCTCAGACGAGCAAG	AGTCACAGACGAACTGCCGAGA
CD81	AAGCAGTTCTATGACCAGGCCCTAC	TGAGGTGGTCAAAGCAGTCAGTG
SR-BI	ATGAAATCTGTCGCAGGCATTG	TGCATCACCTTGGGCATCA
Claudin-1	GCATGAAGTGTATGAAGTGCTTGGA	CGATTCTATTGCCATACCATGCTG
Occludin	AAGAGTTGACAGTCCCATGGCATAC	ATCCACAGGCGAAGTTAATGGAAG
RBBP6	GCGACCTGCAGATCACCAA	TGCCATCGCTGGTTCAGTTC

LDLR, LDL receptor; SR-BI, scavenger receptor class B type I; RBBP6, retinoblastoma binding protein 6.

4 mM $MgCl_2$, 0.5 μM upstream and downstream PCR primers, and 2 μl first-strand cDNA as a template. To control for variations in the reactions, all PCR data were normalized against RBBP6 expression [Chen et al., 2008]. All PCRs were performed in triplicate. The gene expression levels are shown as the relative ratios to those in normal liver and the results are expressed as means ± standard deviation (SD). Continuous variables were compared using the Mann–Whitney *U*-test. Correlations were evaluated by Spearman's rank correlation coefficient. Values of $P < 0.05$ were considered statistically significant.

Liver biopsy samples from patients with chronic hepatitis C (n = 8) and from living donors for liver transplantation (n = 3) were used for immunohistochemical analysis. Each specimen was fixed in formaldehyde, paraffin-embedded, cut into 5-μm thick sections, and dewaxed in xylene. An endogenous peroxidase block was performed with 3% hydrogen peroxide in methanol for 15 min. For antigen retrieval, tissue slides were incubated with Antigen Retrieval Reagent-Basic (R&D Systems, Minneapolis, MN) at 95°C for 5 min. After cooling to room temperature, monoclonal anti-human claudin-1 (final concentration 1.5 μg/ml; Abcam, Tokyo, Japan) and polyclonal anti-human occludin (final concentration 2 μg/ml; Abcam) were used as primary antibodies. Histofine Simple Stain PO (Nichirei, Tokyo, Japan) was used in our assay in accordance with the manufacturer's protocol.

RESULTS

The transcriptional levels of genes related to entry of HCV in the host cell were examined by RT-qPCR in liver samples and compared among 103 patients with chronic hepatitis C (Table I) and 35 healthy individuals. As shown in Figure 1, the transcription of LDLR was markedly suppressed in HCV-infected livers when compared with normal livers ($P < 0.0001$). Expression levels for genes encoding CD81 and SR-BI were comparable between HCV-infected and normal liver samples. Transcription of the claudin-1 was significantly decreased ($P < 0.0001$) and for occludin it was clearly increased in HCV-infected liver samples ($P < 0.0001$). Correlation of the levels of transcription for pairs of genes in HCV-infected and normal livers were evaluated. All pairs with significant correlation are listed in Table III. A strong positive correlation was found between each pair of LDLR, claudin-1 and occludin, and between CD81 and SR-BI, in HCV-infected liver as well as normal liver (Table III, Fig. 2). Serum levels of LCL-C and HCV core antigen showed a significant positive correlation (Fig. 3A). Both serum parameters were negatively correlated with the transcriptional levels of LDLR (Fig. 3B,C), but not with those of other genes examined (data not shown). Formaldehyde-fixed liver samples were incubated with appropriate specific antibodies to ascertain protein expression levels of claudin-1 and occludin. In HCV-infected livers, claudin-1 and occludin were detected and localized to hepatocyte membranes, exhibiting low-level staining. Their expression was not evident in normal liver samples (Fig. 4).

The levels of core antigen varied with genotypes (genotype 1, 7715.84 ± 7774.92 fmol/l; genotype 2, 5917.54 ± 7145.22 fmol/l) but the results described above were independent of HCV genotype or viral quantity (data not shown). Moreover, the results were independent of sex, histological grading, or staging (data not shown).

DISCUSSION

To the best of our knowledge, there have been no human studies regarding the effect of HCV infection on the expression profile of genes associated with viral entry in the liver. Here, we present the results from patients with chronic hepatitis C. Our data were not simply from hepatocytes, because the biopsied liver samples contained other cell types and HCV RNA is often found in lymphocytes [Durand et al., 2010; Stamataki, 2010]. However, hepatocytes were in the majority for the samples and the obtained data were

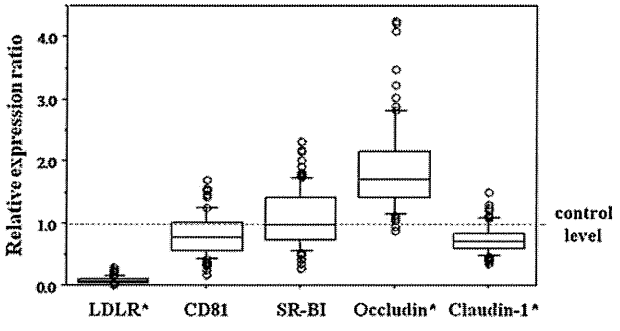

Fig. 1. Transcription levels of genes associated with viral entry in HCV-infected livers. The broken line indicates the mean level in normal control subjects. LDLR, LDL receptor; SR-BI, scavenger receptor class B type I. *Significantly different to normal liver ($P < 0.01$).

TABLE III. Correlations Between Pairs of Genes Expressed in Liver Samples

	Correlation coefficient	*P*-value
HCV-infected liver (n = 103)		
CD81 versus SR-BI	0.652	<0.0001
LDLR versus Claudin-1	0.381	<0.0001
Claudin-1 versus Occludin	0.341	0.0004
LDLR versus Occludin	0.315	0.0012
LDLR versus SR-BI	0.284	0.0036
Normal liver (n = 35)		
LDLR versus Occludin	0.724	<0.0001
Claudin-1 versus Occludin	0.628	<0.0001
CD81 versus SR-BI	0.620	<0.0001
LDLR versus Claudin-1	0.463	0.0051
SR-BI versus Occludin	0.353	0.0372
SR-BI versus Claudin-1	0.337	0.0463

LDLR, LDL receptor; SR-BI, scavenger receptor class B type I.

independent of the grade of lymphocytic infiltration (grading). Therefore, it was considered that the data were representative of those from hepatocytes.

The transcription levels of LDLR were markedly suppressed in HCV-infected livers and were inversely correlated with the serum levels of LDL-C and HCV core protein (Figs. 1 and 3). The levels of LDL-C and HCV core protein were positively correlated (Fig. 3). It has been demonstrated that HCV inhibits the activity of microsomal triglyceride transfer protein (MTP), which results in down-regulation of VLDL secretion, hypobetalipoproteinemia and hypocholesterolemia [Perlemuter et al., 2002; Huang et al., 2007; Syed et al., 2010]. Because HCV virion assembly and secretion utilize the VLDL pathway, and HCV particles are found in the blood as lipoprotein complexes [Huang et al., 2007; Syed et al., 2010], positive correlation between serum levels of HCV core antigen and LDL-C in this study was compatible with previous observations. Suppressed expression of LDLR in HCV-infected livers may reflect intracellular overload of cholesterol and triglyceride, which arises from reduced MTP activity and increased lipogenesis in hepatocytes [Brown and Goldstein, 1981; Goldstein and Brown, 1990; Nakamuta et al., 2009], and it has been observed that expression of LDLR is inversely related to the concentration of LDL-C [Goldstein and Brown, 2009]. Accordingly, the presented results regarding the levels of LDLR, LDL-C, and HCV core antigen confirm the expression profiles described in previous studies.

The expression of other HCV receptors involved in cell entry was also examined. In vitro studies have shown conflicting results in the expression profile of these receptors. For example, one group has reported slightly up-regulated claudin-1 expression [Reynolds et al.,

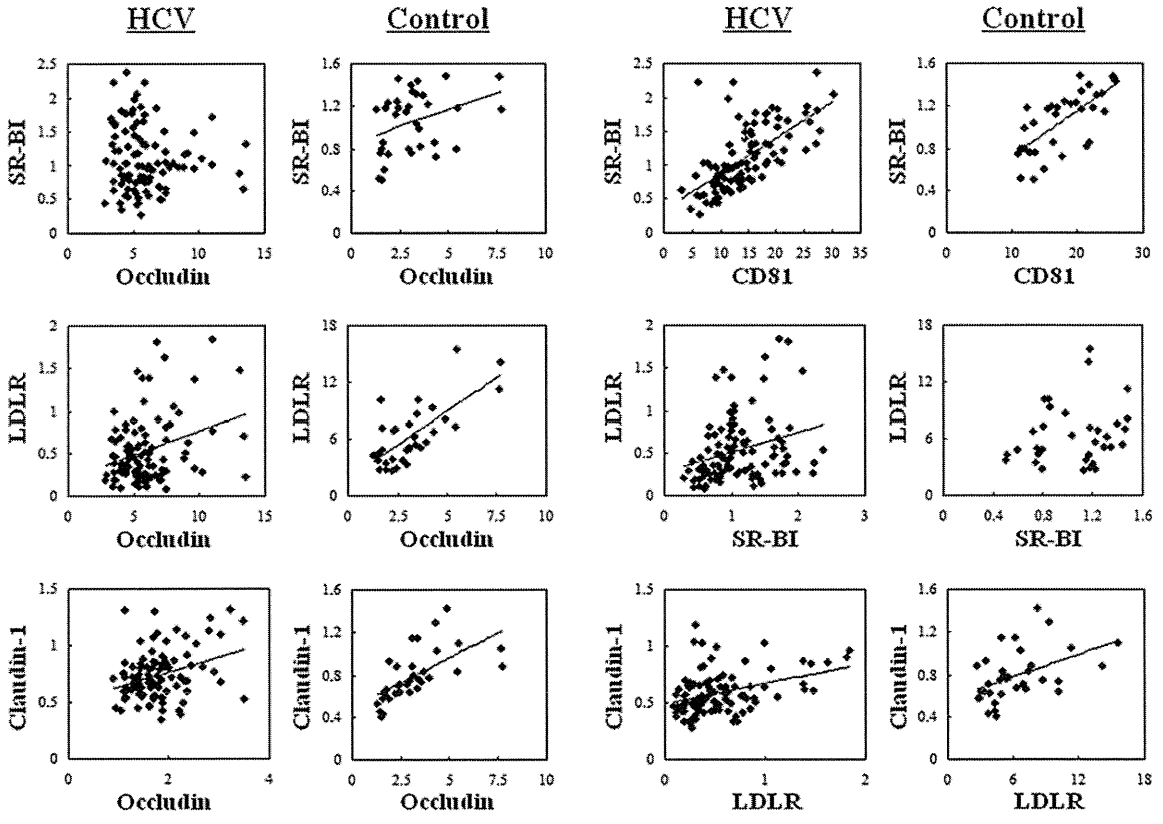

Fig. 2. Correlation analysis of gene expression levels in HCV-infected (HCV) and normal (control) livers. SR-BI, scavenger receptor class B type I; LDLR, LDL receptor.

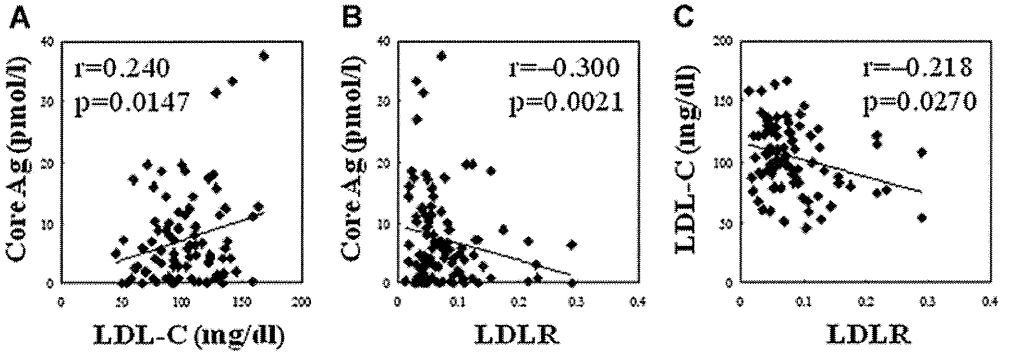

Fig. 3. Correlation of serum levels for LDL-cholesterol (LDL-C) versus HCV core antigen (Core Ag, **A**), gene expression levels of LDL receptor (LDLR) versus serum levels of Core Ag (**B**), and gene expression levels of LDLR versus serum levels of LDL-C (**C**).

2008] and another has shown down-regulated expression of claudin-1 and occludin [Liu et al., 2009] in HCV-infected Huh7.5 cells. The use of cultured cells as a model for infection does not fully represent the situation in the human liver because polarity is crucial to liver function, and Huh7 cells are relatively nonpolarized [Brazzoli et al., 2008]. Reynolds et al. [2008] have reported the immunohistochemical expression of CD81, SR-BI, and claudin-1 in human liver, with no significant changes noted in CD81 and SR-BI expression levels between normal and HCV-infected livers. However, hepatocellular levels of claudin-1 were increased in HCV-infected livers compared with normal livers [Reynolds et al., 2008]. In this study, no significant difference was observed in the expression of CD81 and SR-BI between normal and HCV-infected livers at the transcriptional level (Fig. 1). In terms of tight-junction proteins, the transcriptional levels of the claudin-1 gene were down-regulated and those of the occludin gene were up-regulated in HCV-infected livers as compared to normal livers (Fig. 1). Immunohistochemical staining revealed that expression of claudin-1 and occludin

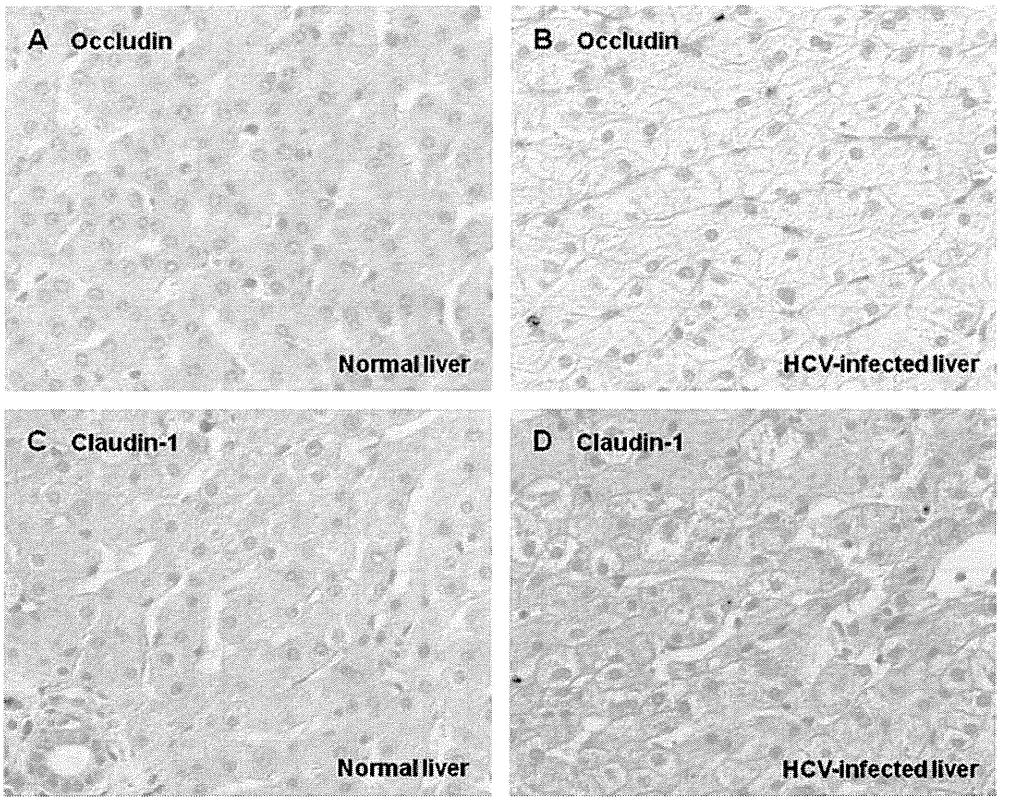

Fig. 4. Immunohistochemical staining of normal (**A**,**C**) and HCV-infected liver tissue (**B**,**D**) for occludin (A,B) and claudin-1 (C,D). ×200 magnification (A,B), ×100 magnification (C,D).