Figure S4. BDCA3+DCs produce various cytokines and IL-28B upon poly IC stimulation, exhibiting suppressive effect on HCV replication.

A. BDCA3+DCs and mDCs were placed at 2.5×104 cells/100 µl and were incubated with 25 µg/ml poly IC, and pDCs with 5µM CPG-DNA. The supernatants were examined for TNF-α, IL-6, IL-10, and IL12p70. Results are shown as mean + SEM from 15 independent experiments. *, $p < 0.05$; **, $p < 0.005$ by Kruskal-Wallis test. n.d., not detected

B. The comparison of the suppressive effect on HCV replication of supernatants from poly IC-stimulated BDCA3+DCs and recombinant IFN-λs. As for an assessment of HCV replication, Huh7 cells transfected with pNNeo/3-5B harboring subgenomic replicon-(HCV-N strain) was used (2). The IL-28B concentration in the supernatants from BDCA3+DCs was determined by ELISA. HCV replicon-positive Huh7 cells were incubated with various concentrations of the supernatants adjusted by IL-28B level or recombinant IL-29 (rIL-29), rIL-28A or, rIL-28B. After 48 hrs, Huh7 cells were harvested and were subjected to real time PCR analysis for HCVRNA quantification as reported previously (2).

HCV RNA levels are shown as relative percentages of the untreated control. For each sample, RT-PCR was performed in triplicate. The mean value obtained from 3 independent experiments is plotted; error bars indicate the SEM.
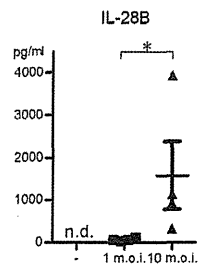
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Figure S5. BDCA3+DCs produced IL-28B in response to HCVcc in an MOI-dependent manner. BDCA3+DCs were incubated for 24h with HCVcc-free medium (as depicted as -), HCVcc at an MOI of 1 or 10. The supernatants were examined for IL-28B. Results are shown as mean + SEM from 4 independent experiments. *, p < 0.05 by paired-t test
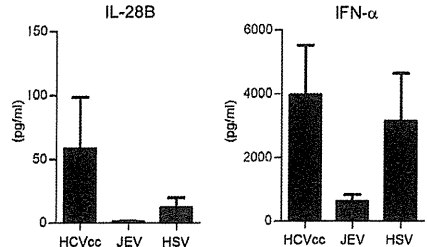
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Figure S6. Plasmacytoid DCs produce IL-28B and IFN-α upon HCVcc stimulation.
Plasmacytoid DCs were cultured at 2.5x104 cells for 24 h with HCVcc, JEV or HSV at an MOI of 10. The levels of IL-28B and IFN-α in the supernatants were measured by ELISA. Results are shown as mean + SEM from 6 experiments.
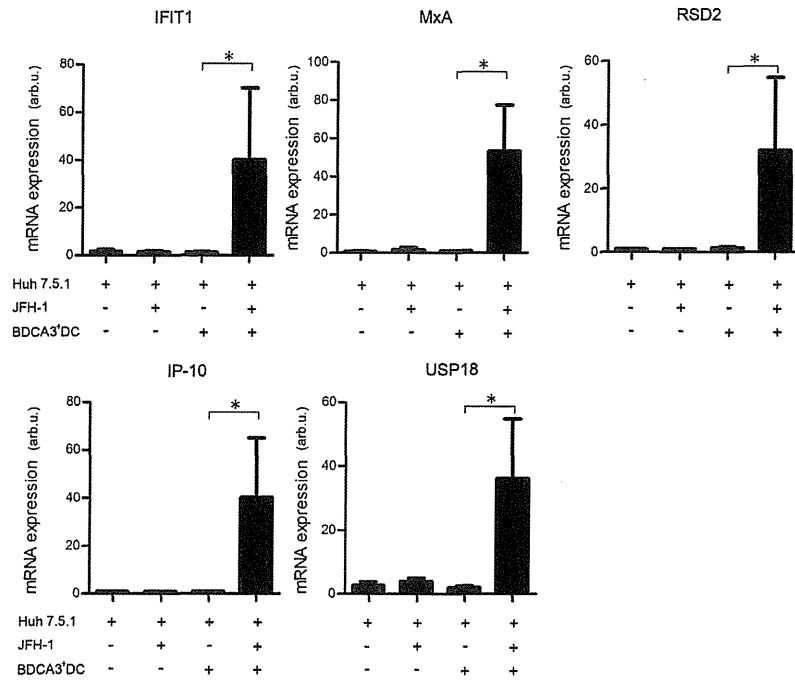
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Figure S7. Various ISGs are induced in JFH-1-transfected Huh7.5.1 cells in the presence of BDCA3+DCs. BDCA3+DCs were co-cultured at 2.5x104 cells with JFH-1-transfected (M.O.I.=2) or –untransfected Huh7.5.1 cells for 24h. After non-adherent BDCA3+DCs were removed by extensive washing the culture wells, Huh7.5.1 cells were harvested and were subjected to real time RT-PCR for the quantification of IFIT1, MxA, RSD2, IP-10 and USP18. The relative mRNA expression (arbitrary unit) was compared using 18S as internal reference. The assays were performed according to the manufacturer's instructions. Results are shown as mean + SEM from 5 experiments. *, $p < 0.05$ by paired-t test

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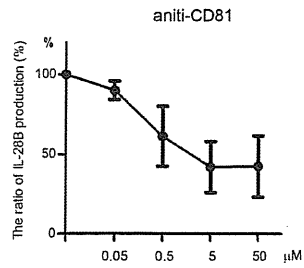Figure S8: Anti-CD81 antibody inhibits HCVcc-induced IL-28B from BDCA3+DCs in a dose-dependent manner.
BDCA3+DCs were incubated for 24h with different concentrations of anti-CD81 antibody. The ratios of IL-28B levels are shown between the samples with various concentration of anti-CD81 antibody and those without. The horizontal bars indicate mean ± SD of 3 experiments.
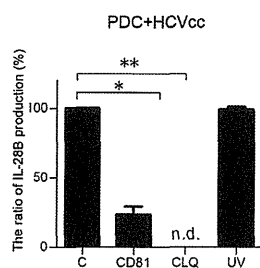
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Figure S9: The CD81 and endosome acidification is involved in the production of IL-28B from HCV-stimulated pDCs, but HCV replication is not necessary.
Plasmacytoid DCs were cultured at 2.5 x 104 cells with HCV at an MOI of 10. As the same as the experiments with BDCA3+DCs, UV-irradiated HCVcc, the treatments with anti-CD81Ab (5µg/ml) or chloroquine (10µM) were performed. The supernatants were examined for IL-28B. Results are expressed as ratios of IL-28B quantity between samples with or without the treatments. The values are shown as mean + SEM from 5 independent experiments. *, p < 0.05 by paired-t test
C, UV, CD81, CLQ, see Figure 5. n.d, not detected

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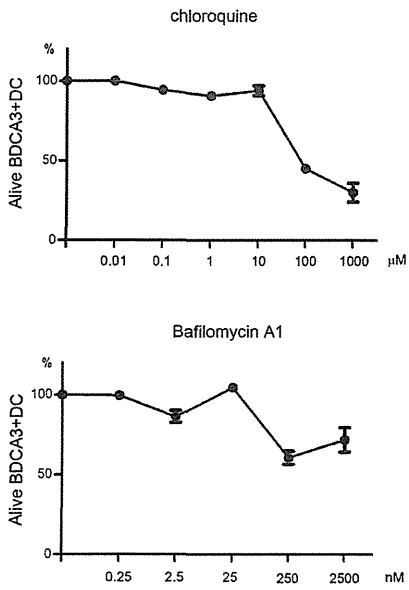
Figure S10. The relationships between concentrations of chloroquine, bafilomycin A1 and the viability of BDCA3+DCs.
BDCA3+DCs were incubated in the presence of different concentrations of chloroquine or bafilomycin A1. After 24h, the viability of BDCA3+DCs was evaluated by a trypan blue dye-exclusion test. The values are expressed as the ratios of live cells in samples with or without the treatments. The horizontal bars indicate means ± SD of 3 experiments.

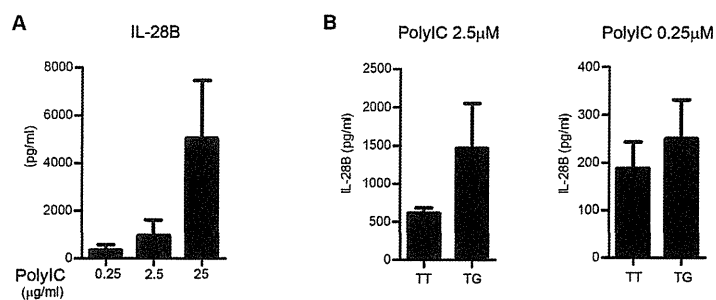173x232mm (300 x 300 DPI)

Figure S11. The quantity of IL-28B from poly IC-stimulated BDCA3+DCs were comparable regardless of the IL-28B genotype, even at the lower concentrations of poly IC.
BDCA3+DCs were incubated for 24h with various concentrations of Poly IC. The levels of IL-28B are quantified by ELISA. The values are shown as mean + SEM from 3 independent experiments.

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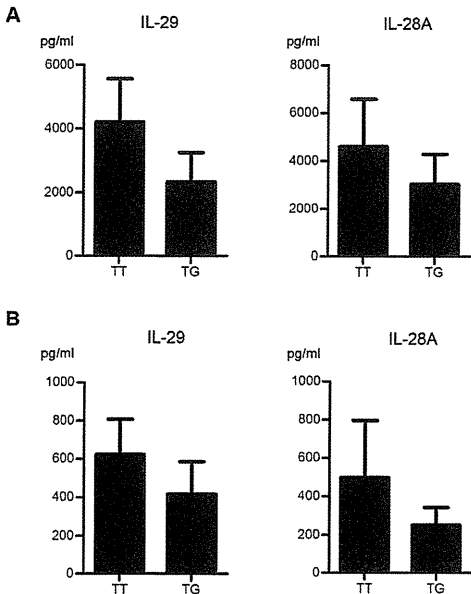
Figure S12. The quantity of IL-29 and IL-28A produced from BDCA3+DCs stimulated with poly IC- or JFH-1-infected Huh 7.5.1 .
BDCA3+DCs of healthy donors with the IL-28B major (rs8099917, TT) or the minor (TG) genotype were cultured at 2.5x104 cells with 25 µg/ml poly IC (A), or with JFH-1-infected- Huh 7.5.1 cells (B) for 24 h. The supernatants were subjected for IL-29 and IL-28A ELISA. The results are the mean + SEM from 15 donors with TT and 8 with TG (A), and from 8 donors with TT and 7 with TG (B), respectively.

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ORIGINAL ARTICLE – HEPATOBILIARY TUMORS

Prognostic Significance of Antithrombin III Levels for Outcomes in Patients with Hepatocellular Carcinoma After Curative Hepatectomy

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ABSTRACT

Background. Although several studies have shown that serum antithrombin III (ATIII) has anti-inflammatory effects, the prognostic value of ATIII in HCC is unknown. We investigated the influence of preoperative ATIII levels on the outcome of patients who underwent hepatectomy for hepatocellular carcinoma (HCC).

Methods. Data from 440 patients (314 patients with ATIII ≥70 % and 126 patients with ATIII <70 %) who underwent curative hepatectomy for HCC were retrospectively collected and analyzed. To overcome bias due to the different distribution of covariates for the 2 groups, propensity score matching was performed on the patients, and outcomes were compared.

Results. The propensity score analysis revealed that 65 patients with ATIII of ≥70 % (group 1) and 65 patients with ATIII of <70 % (group 2) had the same preoperative and operative characteristics (excluding the ATIII level). The overall survival rate and the disease-free survival rate was significantly higher in group 1 than in group 2 ($P = 0.005$ and 0.011, respectively). Multivariate analysis showed that ATIII was a significant favorable factor for overall survival and disease-free survival of patients with HCC after curative hepatectomy.

Conclusions. The prognosis of patients with HCC was found to be associated with preoperative antithrombin III levels. ATIII may be useful for predicting outcomes of patients with HCC after curative hepatectomy.

Hepatic resection is a well-accepted therapy for hepatocellular carcinoma (HCC), but many patients develop cancer recurrence, with the cumulative 5-year HCC recurrence rate being over 60 %.[1,2] A high incidence of tumor recurrence after hepatic resection remains a major drawback. The risk factors for prognosis after resection of HCC have been extensively studied.

Antithrombin III (ATIII) is a heparin-binding protein and a major inhibitor of coagulation proteases, primarily thrombin and factor Xa.[3] ATIII has been reported to efficiently inhibit tumor angiogenesis in a mouse model.[4,5] In clinical settings, decreased plasma ATIII levels have been described in a variety of different cancers, including lung, colon, ovary, and prostate cancers.[6–8] However, few data are available on the impact of ATIII on outcomes of patients with HCC who underwent hepatectomy.

In this study, we aimed to investigate the effect of ATIII on survival and HCC recurrence in patients who underwent curative hepatic resection by both methods of one-to-one match study using propensity score and multivariate analysis.

METHODS

Between the years 2000 and 2008, a total of 440 patients with HCC underwent curative hepatectomy as an initial treatment at the Department of Gastroenterological Surgery, Hiroshima University Hospital, Hiroshima, Japan. The study was approved by the concerned institutional

review boards. Written informed consent was obtained from all patients. The patients were categorized into 2 groups on the basis of their preoperative ATIII level: ≥70 % (group 1, $n = 314$), and <70 % (group 2, $n = 126$).

The type of hepatectomy selected was based on liver function and tumor extent.[9,10] Liver function was assessed by the Child-Pugh classification and the indocyanine green retention rate at 15 minutes (ICG-R15).[11] If the liver function was sufficient, anatomic resection (segmentectomy, sectionectomy, or hemihepatectomy) was performed.[12,13] In patients with insufficient hepatic reserve, limited resection was performed. For example, right hemihepatectomy could be tolerated if the ICG-R15 was in the normal range. One-third of the liver parenchyma could be resected for patients with ICG-R15 of 10–19 %, segmentectomy was possible for patients with ICG-R15 of 20–29 %, and limited resection was possible for patients with ICG-R15 of ≥30 %.[10] Hepatectomy was performed using procedures described by Itamoto et al.[9] Postoperative follow-up included liver function tests, serum alfa-fetoprotein (AFP), hepatic ultrasonography on a 3-month basis, and computed tomographic scans every 6 months. Follow-ups were performed in outpatient clinics or by the patients' general practitioner. Patients with intrahepatic recurrence were managed with ablative therapies such as radiofrequency ablation and percutaneous ethanol injection therapy, transarterial chemoembolization (TACE), or surgery, including living donor liver transplantation. Data were updated until June 2011 and survival was computed from the date of the initial surgery.

Definitions

Normal ATIII is defined as a level of ≥70 % in this study, because it has been shown that anti-thrombin activity of heparin was significantly decreased in the serum ATIII level-dependent manner when plasma ATIII levels were <70 %.[14,15] Major hepatectomy was defined as the resection of 3 or more Couinaud segments. Curative hepatectomy was defined as the removal of all recognizable tumors. All postoperative complications were reviewed for at least 30 days after surgery. The complications were graded according to the method described by Clavien et al.[16] Complications were considered morbid if they were of grade IIIA or greater. Postoperative mortality was defined as any death that occurred within 30 days of surgery.

Receiver-Operating Characteristic (ROC) Analysis

ROC curve analysis was performed to determine the optimal cutoff values for subsequent analyses. Each cutoff value was determined by seeking the most optimal combination of high sensitivity and specificity values, while maintaining the lowest likelihood ratio of a negative test and the highest likelihood ratio of a positive test.

Statistical Analysis

For continuous variables, parametric analyses were performed using Student's t test, and Mann–Whitney U test was used for non-parametric analyses. Categorical variables and postoperative courses were compared using χ^2 tests with Yates correction. The Kaplan–Meier method was used for analyses of overall survival and disease-free survival, whereas comparisons between groups were performed using the log-rank test. For factors determined to be significant for overall and disease-free survival using univariate analysis, we performed multivariate analyses using the Cox proportional hazards model. An initial Cox proportional hazards model was applied to the entire study population to identify poor prognostic predictors. To overcome bias due to the different distribution of covariates among patients from the 2 groups, a one-to-one match was created using propensity score analysis.[17,18] The propensity score represents the probability of each individual patient being assigned to a particular condition in a study given a set of known covariates. Propensity scores are used to reduce selection bias by equating groups on the basis of these covariates and are used to adjust for selection bias in observational studies through matching. Variables entered in the propensity model were age, sex, anti-hepatitis C virus (HCV) antibody, and liver function test including total bilirubin, prothrombin time, ICG-R15 %, albumin, and Child–Pugh classification. Tumor size, number of tumor, vascular invasion, and AFP were used as tumor factors. Operative bleeding, operative time, transfusion, and type of hepatectomy were used as operative factors. The model was then used to obtain a one-to-one match by using the nearest-neighbor matching method.[19,20] Once the matched groups were obtained, overall and disease-free survival analyses were performed within each matched subgroup to assess the influence of preoperative ATIII level on prognosis after adjusting the confounding factors. A difference was considered significant if the P value was <0.05. Statistical analyses were performed using the SPSS statistical software version 16 (Chicago, Illinois, USA).

RESULTS

ROC Curve Analysis for Cutoff Value of ATIII

The optimal cutoff values of ATIII for survival and recurrence were determined by ROC curve analysis, respectively. A cutoff value of survival was 72 % of ATIII

with a sensitivity of 47 % and specificity of 72 %. A cutoff value of recurrence was 69 % of ATIII with a sensitivity of 36 % and specificity of 81 %. ATIII value of 70 % has been chosen as a cutoff level in this study, since normal ATIII is defined as a level of $\geq$70 % (Supplementary Figs. 1 and 2).

Clinicopathological Characteristics and Postoperative Course of the Entire Study Group

Differences between the characteristics of patients in the 2 groups are shown in Table 1. Specifically, patients in group 1 had higher prothrombin time (PT) activity, lower serum bilirubin, lower ICG-R15, lower proportion of patients with Child–Pugh class B, greater maximum tumor diameter, and higher frequency of microvascular invasion. The level of preoperative ATIII in group 1 was significantly higher than that in group 2 (88.4 vs. 59.6 %; $P < 0.001$).

In the entire study population, the overall survival rate of patients in group 1 was significantly higher than that of patients in group 2 ($P < 0.001$): in group 1, the 3- and 5-year overall survival rates were 85.0 and 75.8 %, respectively, whereas in group 2, they were 77.1 and 53.1 %, respectively (Fig. 1a). Furthermore, the disease-free survival rate of patients in group 1 was significantly

higher than that of patients in group 2 ($P < 0.001$): the 1-, 2-, and 3-year disease-free survival rates were 75.3, 60.1, and 48.1 %, respectively, in group 1 and 63.5, 43.6, and 27.4 %, respectively, in group 2 (Fig. 1b). Postoperative complications did not differ between the 2 groups (Table 2). Table 2 shows the patterns of cancer recurrence and the treatment details of the recurrences in both groups. The overall recurrence rate was also significantly lower in group 1 than in group 2 ($P < 0.001$): 53.5 versus 70.6 %. Regarding treatment for HCC recurrence, the proportion of patients in whom repeat hepatectomy was selected for treatment in group 1 tended to be higher than that in group 2 ($P = 0.07$). Furthermore, the proportion of patients in whom living donor liver transplantation was selected for treatment in group 1 was significantly lower than that in group 2 ($P = 0.038$).

Results after Propensity Score Match

The characteristics of propensity score-matched patients are shown in Table 1. Sixty-five of the 314 patients with preoperative ATIII levels $\geq$70 % were matched with 65 of the 126 patients with preoperative ATIII levels <70 % after covariate adjustment. Therefore, 249 patients in group 1 and 61 patients in group 2 were excluded because their propensity scores could not be matched. The study group of 130

TABLE 1 Baseline characteristics and operative data on patients who underwent hepatectomy

Characteristic	Whole study series			Propensity matched series		
	ATIII $\geq$ 70 % ($n = 314$)	ATIII < 70 % ($n = 126$)	P	ATIII $\geq$ 70 % ($n = 65$)	ATIII < 70 % ($n = 65$)	P
ATIII (U/ml)	88.4 ± 13.2	59.6 ± 8.5		82.5 ± 11.2	62.3 ± 6.0	
Age (years)	65.2 ± 10.3	64.6 ± 9.4	0.578	65.2 ± 8.2	64.5 ± 10.1	0.685
Sex (M/F)	238/76	85/41	0.074	44/21	42/23	0.711
Anti-HCV antibody positive	185 (59.2 %)	93 (70.8 %)	0.004	47 (72.3 %)	45 (69.2 %)	0.699
Prothrombin time (%)	90.7 ± 16.1	79.1 ± 15.1	<0.001	85.6 ± 14.1	83.8 ± 11.6	0.424
T-Bil (mg/dl)	0.78 ± 0.31	0.91 ± 0.34	<0.001	0.82 ± 0.29	0.83 ± 0.29	0.831
Albumin (g/dl)	4.00 ± 0.42	3.50 ± 0.45	<0.001	3.72 ± 0.36	3.72 ± 0.33	0.939
ICG-R15 (%)	14.8 ± 8.5	23.6 ± 9.7	<0.001	18.4 ± 10.6	18.5 ± 6.8	0.958
Child–Pugh grade, A/B	303/11	96/30	<0.001	60/5	62/3	0.465
Extent of hepatic resection, major/minor	54/260	13/113	0.069	9/56	8/57	0.794
Operation time (min)	292.8 ± 101.8	283.5 ± 105.1	0.394	277.0 ± 84.3	273.9 ± 76.5	0.585
Blood loss (ml)	380.8 ± 478.4	466.8 ± 633.9	0.123	357.5 ± 415.1	357.8 ± 257.8	0.498
Transfusion	18 (5.7 %)	13 (10.3 %)	0.089	2 (3 %)	2 (3 %)	1
AFP (ng/ml)	4568.3 ± 34234	856.3 ± 3546	0.225	884.7 ± 4657	410.8 ± 994.4	0.788
No. of tumors	1.75 ± 1.88	1.62 ± 0.92	0.44	1.65 ± 1.46	1.63 ± 0.91	0.529
Maximum tumor diameter (mm)	39.7 ± 31.4	32.4 ± 27.7	0.024	33.1 ± 23.3	34.0 ± 24.4	0.407
Vascular invasion	94 (29.9 %)	27 (21.4 %)	0.071	16 (24.6 %)	17 (26.1 %)	0.84

Data are reported for whole study and for the matched study population after propensity score analysis. Continuous variables are expressed as mean ± standard deviation

ATIII anti-thrombin III, *HCV* hepatitis C virus, *T-Bil* total bilirubin, *ICG-R15* indocyanine green retention rate at 15 min, *AFP* alfa-fetoprotein

a
Overall survival rate

P < 0.001

b
Disease-free survival rate

P < 0.001

Years after hepatectomy
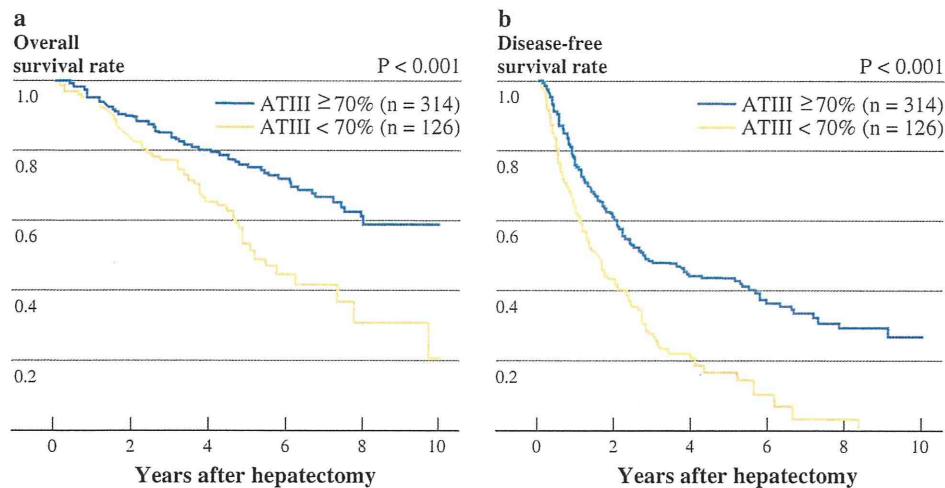
Years after hepatectomy

FIG. 1 Outcomes of the entire study population of 440 patients who underwent liver resection for HCC by stratified with the level of ATIII. **a** Kaplan–Meier curves for the overall survival rate after hepatectomy. Overall survival rates of HCC patients with serum ATIII level of more than 70 IU/ml (group 1, $n = 314$) at 3 and 5 years (85.0 and 75.8 %, respectively) were significantly lower than those of the serum ATIII level of <70 IU/ml (group 2, $n = 126$) at 3 and 5 years (77.1 and 53.1 %, respectively) ($P < 0.001$). **b** Kaplan–Meier curves for the disease-free survival rate after hepatectomy. Disease-free survival rates of group 1 at 1, 2, and 3 years (75.3, 60.1, and 48.1 %) were significantly lower than those of the group 2 at 1, 2, and 3 years (63.5, 43.6, and 27.4 %) ($P < 0.001$)

patients was well matched. In particular, all covariates that significantly affected overall survival in the entire study group were equally distributed over the 2 matched groups. Matched patients in groups 1 and 2 had similar anti-HCV antibody positivity (72.3 vs. 69.2 %; $P = 0.699$), PT activity (85.6 vs. 83.8 %; $P = 0.424$), ICG-R15 (18.4 vs. 18.5 %; $P = 0.958$), serum AFP levels (884.7 vs. 410.8 ng/ml; $P = 0.778$), maximum tumor diameter (33.1 vs. 34.0 mm; $P = 0.407$), number of tumors (1.65 vs. 1.63; $P = 0.529$), and microvascular invasion (24.6 vs. 26.1 %; $P = 0.840$). Other clinical variables and tumor characteristics were also similar in both groups. The preoperative ATIII level of patients in group 1 was significantly higher than that of patients in group 2 (82.5 vs. 62.3 %; $P < 0.001$). The postoperative course of the matched study groups is shown in Table 2. Postoperative complications did not differ between the 2 groups. The mean follow-up period ± standard deviation of groups 1 and 2 was 37.8 ± 36.2 and 34.3 ± 31.0 months, respectively. The overall survival rate of patients in group 1 was significantly higher than that of patients in group 2 ($P = 0.005$): in group 1, the 3-, and 5-year overall survival rates were 92.5 and 83.4 % respectively, whereas in group 2, they were 75.8 and 57.1 %, respectively (Fig. 2a). Furthermore, the disease-free survival rate of patients in group 1 was significantly higher than that of patients in group 2 ($P = 0.012$): the 1-, 2-, and 3-year disease-free survival rates were 74.3, 52.6, and 37.0 %, respectively, in group 1 and 55.6, 43.0, and 29.1 %, respectively, in group 2 (Fig. 2b).

Table 2 shows the patterns of cancer recurrence and the treatment details of the recurrences in both groups. The overall recurrence rate in group 1 tended to be lower than that of group 2 (58.5 vs. 73.8 %; $P = 0.064$). Regarding treatment for HCC recurrence, the proportion of patients in whom repeat hepatectomy was selected for treatment in group 1 tended to be higher than that in group 2 ($P = 0.093$). Furthermore, the proportion of patients in whom TACE was selected for treatment in group 1 was significantly lower than that in group 2 ($P = 0.041$).

Table 3 shows the results from the univariate and multivariate analyses of prognostic factors for overall survival in the whole study. Factors found to be significant in the univariate analysis were PT activity, serum ATIII level, serum albumin level, Child–Pugh grade, extension of hepatectomy, operation time, transfusion, serum AFP level, multiple tumors, tumor size, and microscopic vascular invasion. Multivariate analysis revealed that PT activity, serum ATIII level, serum AFP level, multiple tumors, and microscopic vascular invasion were the independent prognostic factors of overall survival. Table 4 shows the results from the univariate and multivariate analyses of prognostic factors for disease-free survival in the whole study. Factors found to be significant in the univariate analysis include HCV antibody, PT activity, serum ATIII level, serum total bilirubin level, serum albumin level, ICG-R15, Child–Pugh grade, operation time, serum AFP level, multiple tumors, and microscopic vascular invasion. Multivariate analysis revealed that PT activity, serum

TABLE 2 Follow-up data including postoperative complications after curative hepatectomy

Characteristic	Whole study series			Propensity matched series		
	ATIII (≥70 U/ml) (n = 314)	ATIII (<70 U/ml) (n = 126)	P	ATIII (≥70 U/ml) (n = 65)	ATIII (<70 U/ml) (n = 65)	P
Mean follow-up duration (years)	4.09 ± 2.87	3.55 ± 2.30		4.3 ± 2.56	3.46 ± 2.21	
Operative complications						
Clavien–Dindo grade[a]			0.3			1
IIIa	12 (3.8 %)	6 (4.8 %)		2	2	
IIIb	4 (1.3 %)	2 (1.6 %)		0	0	
IVa	0	2 (1.6 %)		0	0	
IVb	2 (0.6 %)	0		0	0	
V	1 (0.3 %)	1 (0.8 %)		0	0	
90-day mortality	10 (3.2 %)	5 (4.0 %)	0.68	2 (3.1 %)	4 (6.2 %)	0.4
Overall recurrence	168 (53.5 %)	89 (70.6 %)	<0.001	38 (58.5 %)	48 (73.8 %)	0.064
First recurrence time (years)	1.83 ± 1.76	1.59 ± 1.57	0.28	1.63 ± 1.40	1.43 ± 1.46	0.52
Recurrence pattern[a]						
Intrahepatic	130 (41.4 %)	81 (64.3 %)	<0.001	34 (52.3 %)	44 (67.7 %)	0.073
Single	58 (18.5 %)	40 (31.7 %)	0.002	14 (21.5 %)	23 (35.4 %)	0.08
Multiple	72 (22.9 %)	41 (32.5 %)	0.037	20 (30.8 %)	21 (32.3 %)	0.85
Extrahepatic	38 (12.1 %)	8 (6.3 %)	0.075	4 (6.2 %)	4 (6.2 %)	1
Main treatment for first recurrence[b]						
Hepatectomy	43 (25.6 %)	14 (15.7 %)	0.07	16 (42.1 %)	12 (25.0 %)	0.093
RFA	28 (16.7 %)	20 (22.5 %)	0.256	7(18.4 %)	10 (20.8 %)	0.78
PEI	7 (4.2 %)	3 (3.4 %)	0.754	4 (10.5 %)	3 (6.3 %)	0.471
TACE	55 (32.7 %)	36 (40.4 %)	0.546	6 (15.8 %)	17 (35.4 %)	0.041
LDLT	2 (1.2 %)	5 (5.6 %)	0.038	1 (2.6 %)	1 (2.1 %)	0.867
Other	22 (13.1 %)	5 (5.6 %)	0.063	1 (2.6 %)	3 (6.3 %)	0.429
No treatment	11 (6.5 %)	6 (6.7 %)	0.953	0	2 (4.2 %)	0.203

ATIII serum antithrombin III, *RFA* radiofrequency ablation, *PEI* percutaneous ethanol injection, *TACE* transcatheter arterial chemoembolization, *LDLT* living donor liver transplantation

[a] Data are expressed as the number of patients (percentage of total patients)

[b] Data are expressed as the number of patients (percentage of patients with recurrence)

ATIII level, serum AFP level, multiple tumors, and microscopic vascular invasion were the independent prognostic factors of overall survival.

DISCUSSION

To our knowledge, this is the first study that investigates the influence of ATIII on HCC patients using propensity score analysis. The present study demonstrated that when other prognostic variables were appropriately adjusted for, overall and disease-free survival after hepatectomy was significantly prolonged in HCC patients with high preoperative levels of ATIII. Therefore, a low preoperative level of ATIII may be considered a risk factor for tumor recurrence and prognosis. The results of this study are in agreement with certain studies, which showed that a decrease in plasma ATIII levels was a risk factor for tumor

recurrence and prognosis in patients with several cancers including lung, colon, ovary, and prostate cancers.[6–8]

The serpin ATIII controls a number of important coagulation enzymes, including factor Xa and thrombin, with the aid of its cofactor, heparin. Heparin activates antithrombin by inducing conformational changes in the protein that specifically enhances binding. While the classical function of ATIII is of an anticoagulant regulator of blood clotting proteinases such as thrombin, recent studies demonstrate its ability to attenuate inflammatory responses by inhibiting cytokines and other inflammatory mediators found within serum and tissue.[3] ATIII has also been reported to suppress the invasion and metastasis of several cancers. Recent studies by Kurata et al.[21] indicate that ATIII prevented hepatic ischemia/reperfusion-induced metastasis of colon cancer cells in a rat model by blocking tumor necrosis factor alpha production. Macrophage inhibitory factor (MIF) has been known to be associated
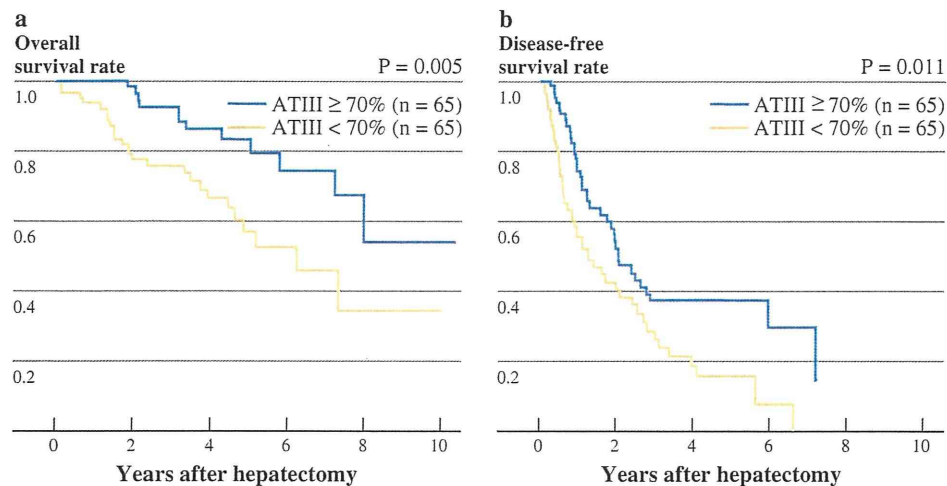
FIG. 2 Outcomes of the matched study population of 130 patients who received liver resection for HCC by stratified with the level of ATIII. **a** Kaplan–Meier curves for the overall survival rate after hepatectomy. Overall survival rates of HCC patients with serum ATIII level of >70 IU/ml (group 1, $n = 65$) at 3 and 5 years (92.5 and 83.4 %, respectively) were significantly lower than those of the serum ATIII level of <70 IU/ml (group 2, $n = 65$) at 3 and 5 years (75.8 and 57.1 %, respectively) ($P = 0.005$). **b** Kaplan–Meier curves for the disease-free survival rate after hepatectomy. Disease-free survival rates of group 1 at 1, 2, and 3 years (74.3, 52.6, and 37.0 %) were significantly lower than those of the group 2 at 1, 2, and 3 years (55.6, 43.0, and 29.1 %) ($P = 0.012$)

with some cancer cell proliferation and invasion. ATIII has been identified as an endogenous MIF-binding protein by forming ATIII–MIF complexes, which reduces MIF biological activity.[22] Recent evidence has shown that thrombin contributes to a more malignant phenotype in vivo by activating tumor-platelet adhesion, tumor adhesion to the subendothelial matrix, tumor implantation, tumor growth, and tumor-associated angiogenesis.[23–25] Kaufmann et al.[26] have shown that some HCC cell lines express a thrombin receptor, proteinase-activated receptor (PAR), and a thrombin-induced increase in HCC cell migration by mediating PAR. Rullier et al.[27] have shown that PAR-1 positive tumor cells are found in HCC. These results suggest that ATIII can suppress proliferation and migration of HCC cells by inhibiting thrombin-induced tumor growth and angiogenesis. It has been also shown that the expression of osteopontin is increased significantly in HCC, and is closely associated with poor prognosis, early recurrence, and metastasis.[28,29] Thrombin cleaves osteopontin into 2 fragments of approximately equivalent size. Osteopontin fragments generated by thrombin cleavage enhance proliferation and adhesion of HCC cells through the activation of integrin β-focal adhesion kinase signaling.[30] Thrombin has been shown to contribute to tumor progression in both a coagulation-dependent and coagulation-independent manner.[31] Further basic and clinical studies are needed to elucidate the antitumor mechanisms of ATIII.

In this study, repeat hepatectomy rather than TACE was selected as a recurrence treatment in more patients with normal level of ATIII, while more patients with decreased level of ATIII underwent TACE rather than hepatectomy for recurrence. This result was thought to be due to high occurrence of early recurrence within 1 year of surgery in patients with decreased level of ATIII. Many cases of recurrence within 1 year after primary hepatectomy are thought to be intrahepatic metastasis from the primary HCC, and survival rate in patients with early recurrence showed worse outcome.[32,33] In our series, most patients who had early recurrence within 1 year of primary hepatectomy were unlikely to receive repeat hepatectomy. Portolani et al.[2] have reported that curative treatment including surgery, percutaneous ethanol injection, and radiofrequency ablation, was feasible in 29.3 % in the early recurrence, while it was 67.6 % in the late recurrence: the proportion of patients who underwent curative treatment for HCC recurrence was significantly higher in the late recurrence than in the early recurrence ($P < 0.05$).

In this study, we have chosen the cutoff for ATIII based on the lower limit of normal level. The optimal cutoff values of ATIII for survival and recurrence determined by ROC curve analysis were 69 and 72 %, respectively. These results indicate that the cutoff value of ATIII with 70 % is valid in this study, and these results are consistent with that normal ATIII is defined as a level of ≥ 70 %.

Before matching by using the propensity score, the clinical characteristics of the entire study population that can strongly influence outcomes differed significantly between the 2 groups. The proportion of patients with better liver function was higher in group 1 than in group 2, and the proportion of patients with advanced HCC also

TABLE 3 Univariate and multivariate analysis of predictive variables of overall survival in the whole study

Variable	Univariate analysis		Multivariate analysis		
	5-year survival rate (%)	P	Hazard ratio	95 % CI	P
Age					
≥70 (n = 167); <70 (n = 273)	68.2; 69.7	0.959			
Gender					
Male (n = 323); female (n = 117)	69.9; 68.9	0.646			
Anti-HCV antibody					
Positive (n = 279); negative (n = 161)	68.5; 70.1	0.357			
Prothrombin time (%)					
≥80 (n = 307); <80 (n = 133)	77.0; 56.7	<0.001	1.62	1.069–2.457	0.023
ATIII (%)					
≥70 (n = 314); <70 (n = 126)	75.8; 53.1	<0.001	1.596	1.012–2.516	0.044
T-Bil (mg/dl)					
≤1.0 (n = 307); >1.0 (n = 133)	72.1; 63.0	0.196			
Albumin (g/dl)					
≥4.0 (n = 332); <4.0 (n = 108)	71.7; 61.7	0.048	1.034	0.621–1.719	0.898
ICG-R15 (%)					
>15 (n = 213); ≥15 (n = 227)	73.2; 65.6	0.093	1.088	0.729–1.623	0.681
Child–Pugh grade					
A (n = 399); B (n = 41)	71.9/50.8	0.001	0.792	0.418–1.501	0.475
Extend of hepatic resection					
Major (n = 373); minor (n = 67)	70.5; 61.2	0.023	0.751	0.449–1.283	0.237
Operation time (h)					
≥6 (n = 280); >6 (n = 180)	73.2; 62.5	0.008	0.844	0.553–1.289	0.433
Blood loss (ml)					
<1,000 (n = 34); ≥1,000 (n = 406)	69.9; 60.2	0.065	1.3	0.607–3.402	0.414
Transfusion					
No (n = 409); yes (n = 31)	71.2; 40.0	<0.001	0.641	0.293–1.296	0.122
AFP (ng/ml)					
≤100 (n = 313); >100 (n = 127)	76.2; 53.2	<0.001	0.571	0.417–0.924	0.004
No. of tumor					
Single (n = 286); multiple (n = 154)	77.0; 54.7	<0.001	0.532	0.386–0.824	0.001
Tumor size (5 cm)					
≥5 (n = 365); <5 (n = 75)	70.8; 62.3	0.033	0.917	0.505–1.664	0.776
Vascular invasion					
No (n = 319); yes (n = 121)	76.1; 51.8	<0.001	2.05	1.303–2.901	0.0001

HCV hepatitis C virus, *ATIII* anti–thrombin III, *T-Bil* total bilirubin, *ICG-R15* indocyanine green retention rate at 15 min, *AFP* alfa-fetoprotein

tended to be higher in the group 1 than in the group 2. To overcome bias due to the different distribution of the severity of liver function impairment between the 2 groups, a one-to-one match was created using propensity score analysis. After matching by propensity score, prognostic variables were appropriately handled, and there was no significant difference in prognostic factors excluding ATIII between the 2 matched groups. This study had a limitation related to the small sample size after propensity score matching. Two hundred forty-nine patients in group 1 and

61 patients in group 2 were excluded by propensity score matching, because their propensity scores could not be matched. Thus, further examination with a larger number of patients may be necessary.

Multivariate analysis agreed with that in previous publications, showing that vascular invasion, multiple tumors, and tumor marker such as AFP were independent prognostic factors associated with overall and disease-free survival rates. These results were compatible with previous reports.[34,35] Regarding with liver function, PT activity and

TABLE 4 Univariate and multivariate analysis of predictive variables of disease-free survival in the whole study

Variable	Univariate analysis		Multivariate analysis		
	5-year survival rate (%)	P	Hazard ratio	95 % CI	P
Age					
≥70 (n = 167); <70 (n = 273)	36.4; 39.7	0.377			
Gender					
Male (n = 323); female (n = 117)	36.6; 39.2	0.357			
Anti-HCV antibody					
Positive (n = 279); negative (n = 161)	34.4; 45.9	0.0164	0.728	0.482–1.101	0.133
Prothrombin time (%)					
≥80 (n = 307); <80 (n = 133)	47.5; 20.5	<0.001	1.621	1.074–2.447	0.021
ATIII (%)					
≥70 (n = 314); <70 (n = 126)	45.7; 20.0	<0.001	1.596	1.012–2.516	0.044
T-Bil (mg/dl)					
≤1.0 (n = 307); >1.0 (n = 133)	43.1; 29.1	0.023	1.009	0.677–1.505	0.965
Albumin (g/dl)					
≥4.0 (n = 332); <4.0 (n = 108)	42.3; 27.4	<0.001	1.043	0.630–1.727	0.871
ICG-R15 (%)					
>15 (n = 213); ≥15 (n = 227)	46.9; 30.3	<0.001	1.088	0.729–1.623	0.681
Child–Pugh grade					
A (n = 399); B (n = 41)	40.6; 21.3	0.034	0.518	0.438–1.517	0.518
Extent of hepatic resection					
Major (n = 373); minor (n = 67)	37.5; 43.6	0.349			
Operation time (h)					
≥6 (n = 280); >6 (n = 180)	40.1; 35.8	0.018	0.84	0.559–1.261	0.4
Blood loss (ml)					
<1000 (n = 34); ≥1000 (n = 406)	40.6; 38.4	0.276			
Transfusion					
No (n = 409); yes (n = 31)	41.0; 38.4	0.262			
AFP (ng/ml)					
≤100 (n = 313); >100 (n = 127)	39.4; 27.0	0.004	0.568	0.386–0.836	0.004
No. of tumors					
Single (n = 286); multiple (n = 154)	43.9; 29.1	<0.001	0.555	0.379–0.811	0.002
Tumor size (5 cm)					
≥5 (n = 365); <5 (n = 75)	37.7; 48.0	0.372			
Vascular invasion					
No (n = 319); yes (n = 121)	76.1; 39.2; 28.751.8	0.004	2.031	1.368–3.015	0.0001

HCV hepatitis C virus, *ATIII* anti–thrombin III, *T-Bil* total bilirubin, *ICG-R15* indocyanine green retention rate at 15 minutes, *AFP* alfa-fetoprotein

ATIII level were significant factors in multivariate analysis. Operative variables such as extension of hepatectomy, blood loss, and transfusion, were associated with poor outcomes in univariate analyses, but these factors were not significant factors in the multivariate analysis.

In conclusion, one-to-one matching study using propensity scores and multivariate analysis showed that the ATIII level was associated with favorable outcomes in HCC patients after curative hepatectomy. ATIII may be useful for predicting outcomes of patients with HCC after curative hepatectomy.

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Safety and Feasibility of Diet-Treated Donors With Steatotic Livers at the Initial Consultation for Living-Donor Liver Transplantation

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Background. The purpose of this study was to evaluate both safety of diet-treated donors and the feasibility of their use for living-donor liver transplantation (LDLT).

Methods. A total of 128 living donors were enrolled in this study between April 2003 and March 2010. Of them, 41 were diagnosed with hepatic steatosis at the initial consultation. Donor selection was based on the findings of liver biopsy accompanied with normalization of liver function tests after diet treatment consisting of an 800 to 1400 kcal/day diet and a 100 to 400 kcal/day exercise without drug treatment, targeting body mass index of 22 kg/m^2.

Results. Body mass index of diet-treated donors was significantly reduced with diet from 23.3±0.6 to 21.9±0.4 kg/m^2 ($P<0.0001$). Liver function tests associated with fatty liver, including alanine aminotransferase, gamma-glutamyl transpeptidase, and total cholesterol levels, also improved with diet ($P=0.0128$, 0.0016, and 0.0004, respectively). The liver biopsy results of most of these donors showed stage 0/1 fibrosis and minimal/mild steatosis after the diet therapy. Surgical outcomes, including postoperative liver function tests, perioperative complications, and liver regeneration rates, did not significantly differ between nondiet-treated and diet-treated donors. Surgical outcomes and the overall survival did not significantly differ between recipients of grafts from nondiet-treated and diet-treated donors.

Conclusion. The use of diet-treated donors for living-donor liver transplantation is feasible with respect to donor safety and the outcome of the recipient when strict selection criteria are used.

Keywords: Diet, Steatosis, Living donor liver transplantation, Biopsy, Fatty liver.

Liver transplantation is the only treatment option for patients with end-stage liver disease. However, the shortage of organs remains a serious problem, and annual death rates per 1000 patient-years at risk is 113.6 while on the waiting list (United Network for Organ Sharing at www.unos.org. accessed in May 2009). Many liver transplantation centers have been forced to modify their criteria for acceptable donors to increase the donor pool. A modified extended criteria donor has been applied to deceased-donor liver transplantation (DDLT), including older donors, donors with prolonged ischemia, donation after cardiac death, those with liver infected with certain viruses, obese donors, and those with steatotic (fatty) livers (*1*).

Implantation of donor livers with severe fatty infiltration is frequently associated with a high incidence of severe ischemic damage, resulting in primary dysfunction and/or primary nonfunction after DDLT (*2–6*).

Meanwhile, living-donor liver transplantation (LDLT) has been accepted and established as an alternative to DDLT (*7*) since it was first successfully performed in 1989 (*8*). Soejima et al. (*9*) described the feasibility of using a steatotic graft even in LDLT with respect to primary nonfunction and reported the effectiveness of short-term treatments consisting of a protein-rich diet, exercise, and bezafibrate for 2 to 8 weeks for donors with a fatty liver (*10*). The obvious advantages of LDLT are the reduction in the mortality of patients on the transplant waiting list and the provision of sufficient preparation time, which is a great merit in scheduling the transplantation (*11, 12*). In our institute, a candidate of living donors with a fatty liver at the initial