Table 4-1. Pathway Analysis of Targeted Genes by miRNAs that Were Commonly Repressed in CH-B, CH-C, HCC-B, and HCC-C Compared with Normal Liver (Cluster 1)

No.	Pathway Name	*P* Value
Down-regulated miRNA in CH-B,HCC-B,CH-C and HCC-C (possibly up-regulating target genes)		
1	Cell adhesion_Platelet-endothelium-leukocyte interactions	1.11E-02
2	Cell cycle_S phase	2.18E-02
3	Protein folding_Protein folding nucleus	2.43E-02
4	Cell cycle_G1-S	3.07E-02
5	Development_Cartilage development	3.89E-02
6	Protein folding_Folding in normal condition	3.89E-02
7	Proteolysis_Connective tissue degradation	3.99E-02
8	Proteolysis_Proteolysis in cell cycle and apoptosis	4.31E-02
9	Signal Transduction_BMP and GDF signaling	5.81E-02
10	Immune_Antigen presentation	6.05E-02

lyzed via cDNA microarray to examine whether the differentially expressed miRNAs could regulate their target genes. Because the absolute standard of miRNA is not available at present, and miRNA expression was compared within the samples and genes analyzed in this study, there might be possible errors when a larger number of samples and genes were analyzed.

Using these systems, we found that the expression profile in miRNAs was clearly different according to HBV and HCV infection for the first time. The differences were confirmed by the nonsupervised learning method, hierarchical clustering (Fig. 2A), and supervised learning methods based on SVM at an 87% accuracy ($P < 0.001$) (Table 2-1). As similarly described, the expression profile in miRNAs was significantly different according to the progression of liver disease (normal, CH, and HCC) in this study. The present CH and HCC expression data were derived from the same patient, and some microarray analyses suggested that the noncancerous liver tissue can predict the prognosis of HCC.[19,20] We examined whether the miRNA expression of paired samples was similar or independent using the Dunnett test[12] (Supplementary Data). Our data indicated that miRNA expression profiling was more dependent on the disease condition than on the paired condition, although the issue of paired samples should be taken into account carefully.

Binary tree prediction analysis and detailed assessment of hierarchical clustering revealed two types of differential miRNAs, one associated with HBV and HCV infection, the other associated with the stages of liver disease that were irrelevant to the differences in HBV and HCV infection. We found that differences in miRNA expression between liver tissues with HBV and HCV (HBV/HCV) were strongly correlated with those in miRNA between cultured cell models of HBV and HCV infection (HBV/HCV) ($r = 0.73$ $P = 0.0006$) (Fig. 5). Thus, there exist HBV- and HCV-infection–specific miRNAs that potentially regulate viral replication and host gene signaling pathways in hepatocytes.

Table 4-2. Pathway Analysis of Targeted Genes by Differentially Expressed miRNAs Between HBV-Related Liver Disease (CH-B,HCC-B) and HCV Related Liver Disease (CH-C,HCC-C Cluster 2)

No.	Pathway Name	*P* Value
Down-regulated miRNA in CH-C,HCC-C (possibly up-regulating target genes)		
1	Immune_Phagosome in antigen presentation	5.80E-04
2	Muscle contraction	1.05E-03
3	Immune_Antigen presentation	5.75E-03
4	Cell cycle_Meiosis	1.49E-02
5	Reproduction_Male sex differentiation	2.06E-02
6	Cell adhesion_Platelet aggregation	2.77E-02
7	Transport_Synaptic vesicle exocytosis	3.56E-02
8	Inflammation_Kallikrein-kinin system	3.73E-02
9	Inflammation_IgE signaling	4.10E-02
10	Development_Skeletal muscle development	5.02E-02
Down-regulated miRNA in CH-B,HCC-B (possibly up-regulating target genes)		
1	Signal Transduction_Cholecystokinin signaling	1.15E-04
2	Inflammation_NK cell cytotoxicity	5.29E-03
3	Signal transduction_CREM pathway	5.31E-03
4	Reproduction_GnRH signaling pathway	7.80E-03
5	DNA damage_DBS repair	1.02E-02
6	Cell cycle_G2-M	1.63E-02
7	Development_Neuromuscular junction	2.07E-02
8	Apoptosis_Apoptosis mediated by external signals	2.42E-02
9	Reproduction_FSH-beta signaling pathway	2.92E-02
10	Cell adhesion_Amyloid proteins	3.81E-02

Table 4-3. The Pathway Analysis of Targeted Genes by Differentially Expressed miRNAs Between CH and HCC (Cluster 3)

No.	Pathway Name	*P* Value
	Down-regulated miRNA in HCC (possibly up-regulating target genes)	
1	Cytoskeleton_Spindle microtubules	2.15E-03
2	Transcription_Chromatin modification	5.27E-03
3	Proteolysis_Ubiquitin-proteasomal proteolysis	6.43E-03
4	Cell adhesion_Cell-matrix interactions	7.30E-03
5	Cell cycle_Meiosis	7.83E-03
6	DNA damage_Checkpoint	1.69E-02
7	Reproduction_Progesterone signaling	1.94E-02
8	Apoptosis_Apoptotic mitochondria	3.14E-02
9	Translation_Regulation of initiation	4.22E-02
10	Signal transduction_WNT signaling	4.26E-02
	Up-regulated miRNA in HCC (possibly down-regulating target genes)	
1	Inflammation_IgE signaling	1.05E-02
2	Inflammation_Kallikrein-kinin system	2.46E-02
3	Inflammation_Innate inflammatory response	2.51E-02
4	Inflammation_Histamine signaling	4.25E-02
5	Inflammation_Neutrophil activation	4.55E-02
6	Chemotaxis	4.68E-02
7	Inflammation_IL-12,15,18 signaling	5.16E-02
8	Inflammation_NK cell cytotoxicity	7.25E-02
9	Cell cycle_G0-G1	7.53E-02
10	Inflammation_Complement system	7.72E-02

	HBV/HCV	
	Tissue	Huh7.5
hsa-miR-20	0.61	0.36
hsa-miR-23a	-1.12	-1.3
hsa-miR-27a	-0.75	-1.51
hsa-miR-34c	-2.29	N.D.
hsa-miR-105	2.13	N.D.
hsa-miR-124b	-1.63	-10.54
hsa-miR-133b	1.28	-3.64
hsa-miR-134	2.52	-0.63
hsa-miR-142-5p	-1.34	-4.39
hsa-miR-151	0.86	-0.29
hsa-miR-182*	1.16	0.37
hsa-miR-190	1.04	1.32
hsa-miR-191	0.48	1.16
hsa-miR-193	0.74	-0.03
hsa-miR-194	0.74	0.76
hsa-miR-211	4.68	5.26
hsa-miR-324-5p	0.59	1.16
hsa-miR-340	0.57	1.68
hsa-let-7a	-1.14	-4.51

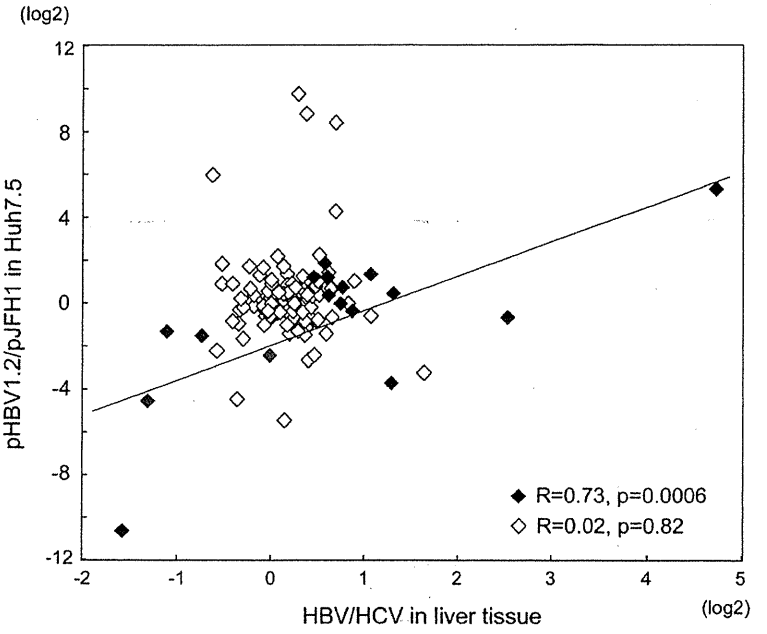

Fig. 5. Correlation between differences in miRNA expression between liver tissues infected with HBV and HCV and those in miRNA expression between cultured cell models of HBV and HCV infections. A total of 140 of 188 miRNAs were confirmed to be expressed in Huh7.5 cells. There was a significant correlation of infection-associated miRNA (closed lozenge) in vitro and in vivo ($r = 0.73$, $P = 0.0006$), but none for the other 121 miRNAs (open lozenge) ($r = 0.02$, $P = 0.82$).

The pathway analysis of targeted genes by miRNAs revealed that 13 miRNAs exhibiting a decreased expression in the HCV group regulate genes related to immune response, antigen presentation, cell cycle, proteasome, and lipid metabolism. Six miRNAs showing a decreased expression in the HBV group regulate genes related to cell death, DNA damage and recombination, and transcription signals. These findings reflected differences in the gene expression profile between CH-B and CH-C as described.[10] Many of the miRNAs were down-regulated in the HCV group rather than in the HBV group. It has been reported that human endogenous miRNAs may be involved in defense mechanisms, mainly against RNA viruses.[21] On the other hand, it is suggested that endogenous miRNAs may be consumed and reduced by defense mechanisms, especially those against RNA viruses.

Although the expressions of these HBV- and HCV-infection–specific miRNAs were irrelevant to the differences in CH and HCC (Fig. 3, cluster 2), some of them have been reported to play pivotal roles in the occurrence of cancer. For example, has-let-7a regulates ras and c-myc genes,[22] and has-miR-34 is involved in the p53 tumor suppressor pathway.[23] These miRNAs were down-regulated in the HBV group, possibly participating in a more aggressive and malignant phenotype in HCC-B rather than in HCC-C. High expression of has-miR-191 was shown to be significantly associated with the worse survival in acute myeloid leukemia,[24] and has-miR-191 was overexpressed in the HBV group compared with the HCV group. On the other hand, has-miR-133b, which was reported to be down-regulated in squamous cell carcinoma,[25] was repressed in the HCV group compared with the HBV group. Some hematopoietic-specific miRNAs such as has-miR-142-5p were up-regulated in the HCV group. Therefore, these miRNAs were not only HBV and HCV infection–associated but also tumor-associated. These findings indicate different mechanisms of development of HCC infected with HBV and HCV (Fig. 6).

Following HCC development, common changes in miRNA expression between HCC-B and HCC-C appeared (Fig. 3, cluster 3). The 23 miRNAs mentioned above clearly differentiated CH and HCC that were irrelevant to the differences in HBV and HCV infections. Seventeen miRNAs were down-regulated in HCC, which up-regulated cancer-associated pathways. Six miRNAs were up-regulated in HCC that down-regulated all inflammation-mediated signaling pathways, potentially reflecting impaired antitumor immune response in HCC. These results suggest that common signaling pathways are involved in HCC development from CH, and that HBV- and HCV-specific miRNAs participate in generating HCC-specific miRNA expressions (Fig. 6). Therefore, these miRNAs might be good candidates for molecular targeting to prevent HCC occurrence, because they reg-

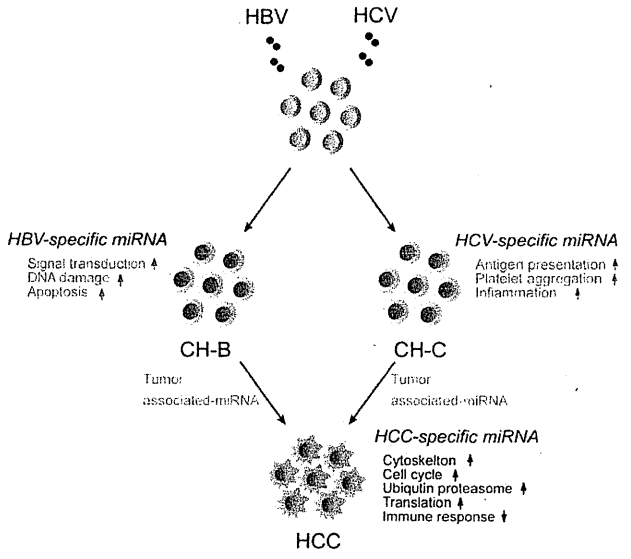

Fig. 6. Infection-associated and HCC-specific miRNAs and liver disease progression.

ulate a common signaling pathway underlying HCC-B and HCC-C development.

In conclusion, we showed that miRNAs are important mediators of HBV and HCV infections as well as liver disease progression. Further studies are needed to enable more detailed mechanistic analysis of the miRNAs identified here and to evaluate the usefulness of miRNAs as diagnostic/prognostic markers and potential therapeutic target molecules.

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ORIGINAL ARTICLE – HEPATOBILIARY TUMORS

Clinical Significance of Alpha-Fetoprotein mRNA in Peripheral Blood in Liver Resection for Hepatocellular Carcinoma

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ABSTRACT

Purpose. Detection of AFP mRNA in peripheral blood is considered a useful predictor of HCC recurrence after resection. However, its interpretation and clinical significance remains to be determined. This study was designed to evaluate the clinical significance of detecting AFP mRNA positive cells in peripheral blood.

Methods. A total of 153 patients without macroscopic vascular invasion, who underwent liver resection, were prospectively enrolled in this study. The pattern of HCC recurrence was confirmed by image studies and divided into four types: (1) no recurrence (control group, $n = 68$); (2) intrahepatic single recurrence (SR group, $n = 28$); (3) intrahepatic multiple recurrences (MR group, $n = 38$); and (4) extrahepatic HCC recurrence (EX group, $n = 19$).

Results. HCC recurrence was identified in 85 (55.6%) patients during a follow-up of 8.6 ± 6.7 (range, 0.7–36) months. Multivariate analysis identified preoperative AFP mRNA (HR = 2.54; $P = 0.006$) as an independent risk factor for HCC recurrence. Preoperative AFP mRNA expression was a significant predictor of HCC recurrence in the MR/EX group ($P = 0.029$) but not in the SR group ($P = 0.467$).

Conclusions. Detection of AFP mRNA expression in peripheral blood before surgery for HCC is a useful predictor of multiple or extrahepatic HCC recurrences.

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Hepatocellular carcinoma (HCC) is the fifth commonest malignant disease and is highly associated with viral hepatitis in up to 90% of cases. Similar to other malignant tumors, HCC has the potential of recurrence with local and distant metastasis. Liver resection has been established as the first-line treatment for HCC, although the high incidence of postoperative recurrence of HCC remains a serious problem. HCC recurrence after liver resection is recognized to have unique characteristics and is divided into three patterns of recurrence: (1) intrahepatic metastasis; (2) multicentric HCC; and (3) extrahepatic metastasis. The diagnosis of these patterns of recurrence requires close follow-up with image studies after liver resection as well as histopathological evaluation of the tumor recurrence, if available.[1]

Circulating tumor cells (CTC) in the peripheral blood or disseminated tumor cells (DTC) in the bone marrow are reported to be the cause of tumor recurrence in various malignant tumors.[2] In liver transplantation for HCC, the fact that the most common site of tumor recurrence is the transplanted allograft provides strong support for this notion and the central role of CTC and DTC in tumor recurrence.[3,4]

The mRNA level of alpha-fetoprotein (AFP) in peripheral blood is a candidate marker of CTC. We reported previously the efficacy of detecting AFP-expressing cells by quantitative RT-PCR in patients who had undergone liver resection or liver transplantation for HCC.[5,6] Despite numbers of publications on this prognostic marker of HCC recurrence, it has not been studied in reference with the patterns of HCC recurrence.

This study was designed to determine the prognostic value of detecting AFP mRNA-positive cells in peripheral blood in patients with HCC who underwent curative resection, in predicting HCC recurrence after surgery, and to clarify the correlation between AFP mRNA expression in peripheral blood and the three patterns of HCC recurrence.

PATIENTS AND METHODS

The study protocol was approved by the Human Subjects Review Committee of Osaka University. All study subjects provided written, informed consent.

Patients

Among 295 consecutive patients who underwent liver resection for HCC between December 2001 and October 2008 in our hospital, 188 patients who underwent curative resection were free of macroscopic portal or venous invasion and consented to this prospective study. Peripheral blood samples (16 ml) were obtained from each participant for analysis of AFP mRNA at the following time points: within 3 days before surgery, and postoperatively immediately after surgery. Of the 188 patients, 37 were excluded because of short follow-up period without HCC recurrence (<12 months), and thus data of 153 patients were subjected to the analysis of risk factors.

The patient demographic and operative data, tumor characteristics, preoperative serum AFP levels, serum levels of protein induced by vitamin K antagonist II (PIVKA-II), and computed tomographic (CT) scans of the abdomen and chest after surgery were collected prospectively. The standard postoperative follow-up consisted of abdominal dynamic CT scan or magnetic resonance imaging (MRI) every 3–4 months with serum AFP, PIVKA-II, and chest X-ray or chest CT scan every 3–6 months. Bone scintigraphy or brain MRI was performed whenever metastasis was suspected.

Patients with HCC > 5 cm in preoperative image studies received transcatheter arterial chemoembolization (TACE) therapy 1–2 months before liver resection. No adjuvant chemotherapy, TACE, or other anticancer treatment was provided to the study patients until HCC recurrence was confirmed.

HCC recurrence confirmed by image studies was divided based on the patterns of the recurrence into: (1) no recurrence (control group); (2) intrahepatic single recurrence after liver resection (SR group); (3) multiple intrahepatic recurrences (MR group); and (4) extrahepatic HCC recurrence (EX group).

Real-Time Quantitative RT-PCR for AFP mRNA in Peripheral Blood

Peripheral blood (16 ml) samples were obtained prospectively from each patient within 3 days before surgery (preoperative AFP mRNA) and again immediately after surgery (postoperative AFP mRNA). The method used for the detection of AFP mRNA in peripheral blood was described previously.[7,8] Briefly, blood samples were collected in a VACUTAINER CPT™ cell preparation tubes with sodium citrate (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 17,000×g for 20 min. The separated mononuclear cells were placed into a 15-ml centrifugation tube, suspended with 10 ml of phosphate buffered saline (PBS), and centrifuged at 2,000 rpm for 10 min. After washing with PBS again, the cells were suspended with TRIzol Reagent (Molecular Research Center, Cincinnati, OH), and stored at −80°C until RNA isolation. AFP mRNA was quantified with the LightCycler™ analysis software (Roche Diagnostics, Mannheim, Germany) using the protocol provided by the manufacturer. The level of AFP mRNA in the blood was expressed relative to that of the mRNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The lower limit of detection of the AFP mRNA by this method was 1.0×10^{-8}, and any value above this level was designated as positive, as described previously.[5,6]

Statistical Analysis

Continuous data were expressed as mean ± standard deviation, and group data sets were compared using the Mann–Whitney U test or Kruskal–Wallis test. Categorical data are presented as percentages, and differences between proportions were compared using the chi-square test. The cumulative risk of HCC recurrence and the 95% confidence intervals (CI) were computed by Kaplan–Meier analysis. Univariate and multivariate risk-factor assessments were performed using the Kaplan–Meier method (log-rank test) and Cox's proportional hazards model. Variables that correlated with the risk of HCC recurrence in the univariate analysis ($P < 0.1$) were entered into the multivariate analysis. $P < 0.05$ was considered significant.

RESULTS

The 153 patients with HCC comprised 116 men and 37 women. The underlying liver disease was HCV ($n = 90$, 58.8%), HBV ($n = 33$, 21.6%), Laennec's ($n = 4$, 2.6%), and no apparent background liver disease ($n = 32$, 20.9%). The mean follow-up duration was 13.4 ± 10.8 (range, 0.4–54.2) months. Of the 153 patients, 68 (44.4%) were recurrence-free after a follow-up period of 22.6 ± 11.3 (range, 12–54.2) months, whereas 85 patients (55.6%) developed HCC recurrence within a follow-up period of 8.6 ± 6.7 (range, 0.7–36) months. The proportion of patients showing each type of recurrence pattern was 44.4% ($n = 68$) for the control group (no recurrence), 16.3% ($n = 28$) for the SR group (intrahepatic single recurrence after liver resection), 24.8% ($n = 38$) for the MR group (multiple intrahepatic recurrences after liver

resection), and 12.4% ($n = 19$) for the EX group (extrahepatic HCC recurrence), which included pulmonary metastasis ($n = 10$, 53%), lymph node metastasis ($n = 3$, 16%), diaphragm metastasis ($n = 3$, 16%), bone metastasis ($n = 2$, 11%), and adrenal gland metastasis ($n = 1$, 5%).

Table 1 shows the demographic and clinical features of the four groups. Age, gender, and background liver disease were similar among the four groups. Tumor size tended to be smaller in the control group and largest in the MR group ($P = 0.018$ between control vs. MR groups). Tumor number was single in 54 of 68 (79.4%)

TABLE 1 Characteristics of patients and hepatocellular carcinoma

	Control group ($n = 68$)	SR group ($n = 28$)	MR group ($n = 38$)	EX group ($n = 19$)	P
Age (years)	65.2 ± 9.9	67.1 ± 9.9	66.6 ± 7.6	63.9 ± 7.8	0.515
Gender (male/female)	46/22	22/6	31/7	17/2	0.157
Primary diagnosis					
HCV	41 (60.3)	16 (57.1)	25 (65.8)	8 (42.1)	0.213
HBV	16 (23.5)	5 (17.9)	5 (13.1)	7 (36.8)	
Laennec's	2 (2.9)	1 (0.4)	0 (0)	1 (5.3)	
Non-B, non-C	14 (20.6)	9 (32.1)	9 (23.6)	5 (26.3)	
Tumor characteristics					
Size (cm)	3.74 ± 2.47	4.14 ± 2.22	5.18 ± 3.63	4.78 ± 3.75	0.055
Number	128 ± 0.67	1.57 ± 1	1.97 ± 1.46	1.8 ± 1.24	0.093
Microscopic vascular invasion (%)	25.4	26	50	26.3	0.06
Histological differentiation (Edmondson classification)					
1	1 (1.8)	1 (3.7)	0 (0)	0 (0)	0.119
2	19 (33.3)	15 (55.6)	12 (31.6)	9 (47.3)	
3	34 (59.6)	10 (37)	25 (65.8)	6 (31.6)	
4	3 (5.3)	1 (3.7)	1 (2.6)	3 (15.8)	
Preoperative TACE (%)	45.5	46.4	47.4	68.4	0.353
Hepatectomy (HR)[a]					
0	34 (50)	17 (60.7)	20 (52.6)	9 (47.4)	0.9
S	8 (11.8)	1 (3.6)	4 (10.5)	3 (15.8)	
1	16 (23.5)	6 (21.4)	7 (18.4)	6 (31.6)	
2	9 (13.2)	4 (14.3)	7 (18.4)	1 (5.3)	
3	1 (1.5)	0 (0)	0 (0)	0 (0)	
Blood loss (ml)	842 ± 1280	647 ± 595	1460 ± 2683	721 ± 454	0.075
Transfusion	6/68 (8.8)	6/28 (21.4)	6/38 (15.8)	0	0.102
Transfused RC-M.A.P. (ml)	133 ± 610	89 ± 253	302 ± 1098	0	0.769
TNM stage[a]					
1	4 (5.9)	4 (14.3)	2 (5.3)	1 (5.3)	0.096
2	50 (73.5)	13 (46.4)	19 (50)	10 (52.6)	
3	12 (17.6)	8 (28.6)	12 (31.6)	5 (26.3)	
4a	2 (2.9)	3 (10.7)	3 (7.9)	3 (15.8)	
4b	0 (0)	0 (0)	2 (5.3)	0 (0)	
AFP (median; range)	17.5 (2–206249)	36.5 (3–31310)	52 (4–179200)	38 (4–947500)	0.314
PIVKA	105 (28–61330)	300 (9–32539)	334 (20–122976)	252 (23–304000)	0.356
AFP mRNA (%)					
Preoperative	4.4	10.7	15.8	10.5	0.264
Postoperative	20.6	42.9	36.8	31.6	0.095
Preoperative and postoperative	4.4	0	5.3	5.3	0.466

Data are mean ± standard deviation or number of patients with percentages in parentheses unless otherwise indicated

RC-M.A.P. Red cell concentrates mannitol adenine phosphate, *AFP* alpha-fetoprotein, *PIVKA* protein induced by vitamin K antagonist, *TACE* transcatheter arterial chemoembolization, *SR* single recurrence, *MR* multiple recurrence, *EX* extrahepatic recurrence

[a] According to the Liver Cancer Study Group of Japan (LCSGJ)

patients of the control group and in 18 of 28 (64.3%) patients of the SR group, whereas a solitary tumor was less frequently seen in 21 of 38 (55%) patients of the MR group and 11 of 19 (58%) patients of the EX group. The number of tumors was the lowest in the control group compared with the MR ($P = 0.007$) and EX ($P = 0.035$) groups. Tumor differentiation according to Edmondson classification, HAI score in background liver, and the extent of liver resection were not different among the four groups. The estimated blood loss and transfused red cell concentrates mannitol adenine phosphate were not significantly different among the groups. AFP and PIVKA-II were not different among the four groups.

The AFP mRNA/GAPDH mRNA ratio in peripheral blood ranged from undetectable and 1.04E−4. AFP mRNA was detected in 14 (9.2%) patients before surgery, whereas 46 (30.1%) patients were positive postoperatively. Six (3.9%) patients were positive for AFP mRNA both preoperatively and postoperatively. A larger proportion of patients of the MR group were AFP mRNA-positive preoperatively and less in the control group than the SR and EX groups, whereas a larger proportion of patients of the SR, MR, and EX groups were AFP-mRNA-positive postoperatively than the control group. The status of AFP mRNA (positive/negative) did not correlate with tumor characteristics, such as microscopic vascular invasion, blood loss, blood transfusion, TNM stage, and PIVKA-II,

TABLE 2 Relationship between preoperative AFP mRNA and various clinical parameters

	Preoperative AFP mRNA		*P*
	Positive ($n = 14$)	Negative ($n = 139$)	
Age (years)	70.1 ± 6.8	65.3 ± 9.2	0.057
Gender (male/female)	11/3	105/34	0.064
Primary diagnosis (%)			
HCV	42.9	60.4	0.203
HBV	14.3	22.3	0.487
Non-B, non-C	42.9	21.6	0.187
Tumor characteristics			
Size (cm)	5.2 ± 3.5	4.2 ± 2.9	0.146
Number	2.57 ± 1.83	1.47 ± 0.92	0.070
Microscopic vascular invasion (%)	30.8	32.4	0.906
Histological differentiation (Edmondson classification)			
1	0 (0)	2 (1.4)	0.947
2	5 (35.7)	50 (36)	
3	8 (57.1)	67 (48.2)	
4	1 (7.1)	7 (5)	
Preoperative TACE (%)	57.1	48.9	0.557
Hepatectomy (HR)[a]			
s	0	16	0.094
0	11	69	
1	0	35	
2	3	18	
3	0	1	
Blood loss (median; range) (ml)	480 (20–16600)	550 (30–2400)	0.724
Blood transfusion (RC-M.A.P.) incidence (amount (ml))	14% (780 ± 736)	10.1% (1539 ± 1781)	0.571
TNM stage[a]			
1	1	10	0.527
2	6	86	
3	6	31	
4a	1	10	
4b	0	2	
AFP (median; range)	396 (4–947500)	32 (2–206249)	0.039
PIVKA	115 (31–304000)	174 (9–122976)	0.917

Data are mean ± standard deviation or number of patients with percentages in parentheses unless otherwise indicated

RC-M.A.P. Red cell concentrates mannitol adenine phosphate, *AFP* alpha-fetoprotein, *PIVKA* protein induced by vitamin K antagonist, *TACE* transcatheter arterial chemoembolization

[a] According to the Liver Cancer Study Group of Japan (LCSGJ)

TABLE 3 Relationship between preoperative AFP mRNA level and clinical parameters

	Preoperative AFP mRNA		*P*
	Positive ratio	Level (mean, range)	
Tumor characteristics			
Microscopic vascular invasion			
Negative	9/103 (8.7%)	4.77E−7 (2.0E−8, 2.42E−06)	0.44
Positive	4/49 (8.2%)	6.17E−7 (2.5E−8, 1.27E−06)	
Edmondson			
1	0/2 (0%)	–	0.499
2	4/55 (7.3%)	6.12E−7 (2.0E−8, 2.42E−06)	
3	8/75 (10.7%)	16.7E−7 (2.47E−8, 1.09E−5)	
4	1/8 (12.5%)	12.7E−7	
Capsule formation			
−	2/22 (9.1%)	1.75E−7 (2.0E−8, 3.29E−7)	0.283
+	12/128 (9.4%)	17.2E−7 (2.4E−8, 1.09E−5)	
TNM stage[a]			
1	1/11 (9.1%)	24.2E−07	0.278
2	6/92 (6.5%)	3.85E−7 (2.0E−8, 1.24E−6)	
3	6/37 (16.2%)	21.4E−7 (4.42E−8, 1.09E−5)	
4a	1/11 (9.1%)	5.45E−8	
4b	0/2	–	
HCC recurrence			
No recurrence	3/68 (4.4%)	36.7E−7 (2.38E−8, 1.09E−5)	0.110
Single recurrence (SR)	3/28 (10.7%)	16.4E−7 (1.24E−6, 2.42E−6)	
Multiple recurrence (MR)	6/38 (15.8%)	2.72E−7 (2.5E−8, 9.05E−7)	
Extrahepatic recurrence (EX)	2/19 (10.5%)	3.7E−8 (2.4E−8, 5.5E−8)	

[a] According to the Liver Cancer Study Group of Japan (LCSGJ)

except it was associated with serum AFP level ($P = 0.039$; Table 2). Furthermore, AFP mRNA expression level did not correlate with tumor characteristics (microscopic vascular invasion, tumor differentiation by Edmondson classification, and capsule formation), TNM stage, or HCC recurrence (Table 3).

Figure 1a shows the recurrence-free survival rate of the SR, MR, and EX groups. The rate was significantly better in the SR group than the MR and EX groups ($P = 0.0015$), and it was almost identical in the latter two groups. Similarly, the survival rate was significantly better in the SR group than the MR and EX groups ($P < 0.0001$; Fig. 1b).

Univariate risk factor analysis showed that tumor size, tumor number, and microscopic vascular invasion were significantly related to HCC recurrence. Preoperative TACE was a candidate risk factor for HCC recurrence ($P = 0.067$); however, it correlated significantly with tumor size ($P < 0.0001$). Serum PIVKA-II level tended to

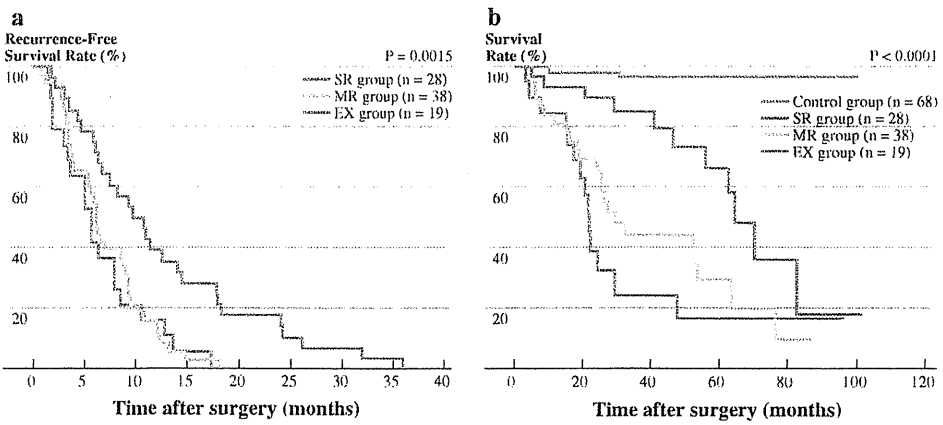

FIG. 1 Kaplan–Meier plot of recurrence-free and overall survival rates after liver resection. **a** There was a significant difference in the recurrence-free survival rate ($P = 0.0015$, log-rank test). **b** There was a significant difference in the overall survival rate ($P < 0.0001$, log-rank test). Control group: no recurrence; SR group: intrahepatic single recurrence after liver resection; MR group: multiple intrahepatic recurrence; EM group: extrahepatic HCC recurrence

be a risk factor for HCC recurrence ($P = 0.076$), whereas AFP was a significant risk factor for HCC recurrence ($P = 0.004$). AFP mRNA expression preoperatively was a risk factor for HCC recurrence ($P = 0.015$), whereas postoperative expression was not ($P = 0.082$). The small subset of both preoperative and postoperative AFP mRNA-positive patients ($n = 6$) did not show any characteristics in terms of incidence in each group (Table 1) and HCC recurrence (data not shown).

Multivariate Cox proportional hazard analysis identified tumor number, preoperative AFP mRNA, microscopic vascular invasion, and serum PIVKA-II level as independent risk factors for HCC recurrence (Table 4).

The HCC recurrence-free survival rate according to AFP mRNA status is shown in Fig. 2a and b. The difference in HCC recurrence-free survival based on preoperative AFP mRNA status was significant, whereas the rate was similar irrespective of the postoperative AFP mRNA status. The difference in the survival rate was more conspicuous when the data of all patients were analyzed (Fig. 2c, d). Patients with positive preoperative AFP mRNA status had significantly worse overall survival than those with negative status, whereas there was no difference in overall survival between patients with positive postoperative AFP mRNA status and those with negative postoperative AFP mRNA status ($P = 0.364$). Among the 85 patients with HCC recurrence, preoperative AFP mRNA was positive in 11 (12.9%) patients. Among 14 patients with positive preoperative AFP mRNA, 11 (78.6%) patients developed HCC recurrence.

We also divided HCC recurrence into two different patterns—solitary intrahepatic recurrence in the SR group, and multicentric or extrahepatic recurrence in the MR/EX group—because the HCC recurrence-free survival rates of the MR and EX groups were almost identical. In the analysis of "time to solitary intrahepatic recurrence," both preoperative and postoperative AFP mRNA statuses did not correlate with HCC recurrence, whereas only preoperative AFP mRNA status correlated significantly with HCC recurrence in the analysis of "multicentric or extrahepatic recurrence" (Fig. 3a–d).

DISCUSSION

The diagnosis of tumor recurrence by detecting circulating tumor cells is already applied in various cancers, such as breast cancer, prostate cancer, and HCC.[2] AFP mRNA has been reported to be a suitable marker for prediction of tumor recurrence, and the efficacy of predicting HCC recurrence after curative resection using AFP mRNA detection has been confirmed in many studies, although others did not.[5,9–14] The reasons for the differences in the utility of AFP mRNA in predicting HCC recurrence are (1) differences in sampling time points among the studies, and (2) differences in RT-PCR technique, using conventional RT-PCR, nested RT-PCR, or quantitative RT-PCR. We consistently used the quantitative RT-PCR method, which is described in detail in several previous studies.[5,6,8]

We reported previously that AFP mRNA is a useful predictor of HCC recurrence in both liver resection and liver transplant patients.[5,6,8] In liver transplant patients, we showed that preoperative detection of AFP mRNA-positive cells in peripheral blood was associated with high incidence of postoperative HCC recurrence, whereas it was not in the anhepatic phase or immediate postoperative period, although the detection rate of AFP mRNA-positive cells in peripheral blood was increased from 9.1 to 30.1%. Based on these results, we hypothesized that the operative maneuver during liver transplantation results in squeezing normal hepatocytes or impotent tumor cells into the blood stream, resulting in detection of AFP mRNA in peripheral blood regardless of the presence of potent CTC.

In the present study, we evaluated the AFP mRNA in a larger population sample of liver resection, limiting the sample to those who underwent complete resection and showed no macroscopic vascular invasion. More importantly, we analyzed these patients with reference to the pattern of HCC recurrence, i.e., SR group, MR group, and EX group, compared with the no recurrence group (control group). The overall recurrence rate in this cohort was 43.2% at 1 year and 63.7% at 3 years, which is similar to the previous report.[1,15]

We reported two major findings in the present study. First, preoperative AFP mRNA was an independent risk factor for HCC recurrence, whereas postoperative AFP mRNA was not. As we pointed out in our previous study of transplant patients, this result supported the notion that detection of AFP mRNA in the immediate postoperative period was not significant. Overall survival was significantly worse in the preoperative AFP mRNA-positive group than mRNA-negative group, whereas it was similar between postoperative AFP mRNA-positive and mRNA-negative groups (Fig. 2b, d), supporting the above-mentioned results. The false positivity of AFP mRNA could simply represent normal hepatocytes or HCC cells being squeezed from the liver into the systemic circulation, and detected by RT-PCR method, rather than representing the complex process of tumor recurrence and systemic spread. This is important because circulating tumor cells are not always viable or proliferative, and most CTCs disappear without causing micrometastasis.[16–19]

The second major finding of the present study was that the recurrence-free survival curve was almost similar for

TABLE 4 Univariate and multivariate risk factor analyses for HCC recurrence

	Relative risk	95% CI	*P*	Relative risk	95% CI	*P*
Age (years)						
≤60	1					
>60	1.13	0.692–1.86	0.619			
Gender						
Male	1					
Female	1.54	0.879–2.68	0.132			
HCV						
−	1					
+	0.92	0.599–1.42	0.712			
HBV (HBsAg+)						
−	1					
+	0.92	0.538–1.56	0.746			
Preoperative TACE						
−	1					
+	1.5	0.97–2.33	0.067			
Tumor size (cm)						
≤5	1			1		
>5	1.64	1.03–2.6	0.037	1.413	0.88–2.28	0.156
Number						
Single	1			1		
Multiple	1.95	1.26–3.01	0.0026	2.07	1.33–3.23	0.001
Microscopic vascular invasion						
−	1			1		
+	1.72	1.1–2.68	0.016	1.83	1.16–2.88	0.009
Histological differentiation (Edmondson classification)						
1, 2	1					
3, 4	1.11	0.72–1.71	0.652			
Capsule formation						
−	1					
+	1.04	0.575–1.88	0.896			
Hepatectomy (HR)[a]						
s, 0	1					
1, 2, 3	0.996	0.64–1.55	0.987			
Blood loss (median; range) (ml)						
≤1000	1					
>1000	1.23	0.51–1.29	0.377			
AFP						
≤20	1					
>20	1.96	1.24–3.01	0.004			
PIVKA-II						
≤200	1			1		
>200	3.58	0.87–14.71	0.076	5.62	1.33–23.8	0.019
Preoperative AFP mRNA						
−	1			1		
+	1.98	1.05–3.73	0.036	2.54	1.31–4.93	0.006
Postoperative AFP mRNA						
−	1			1		
+	1.48	0.95–2.3	0.082	1.37	0.88–2.15	0.166

CI Confidence interval, *AFP* alpha-fetoprotein, *PIVKA* protein induced by vitamin K antagonist

[a] According to the Liver Cancer Study Group of Japan (LCSGJ)

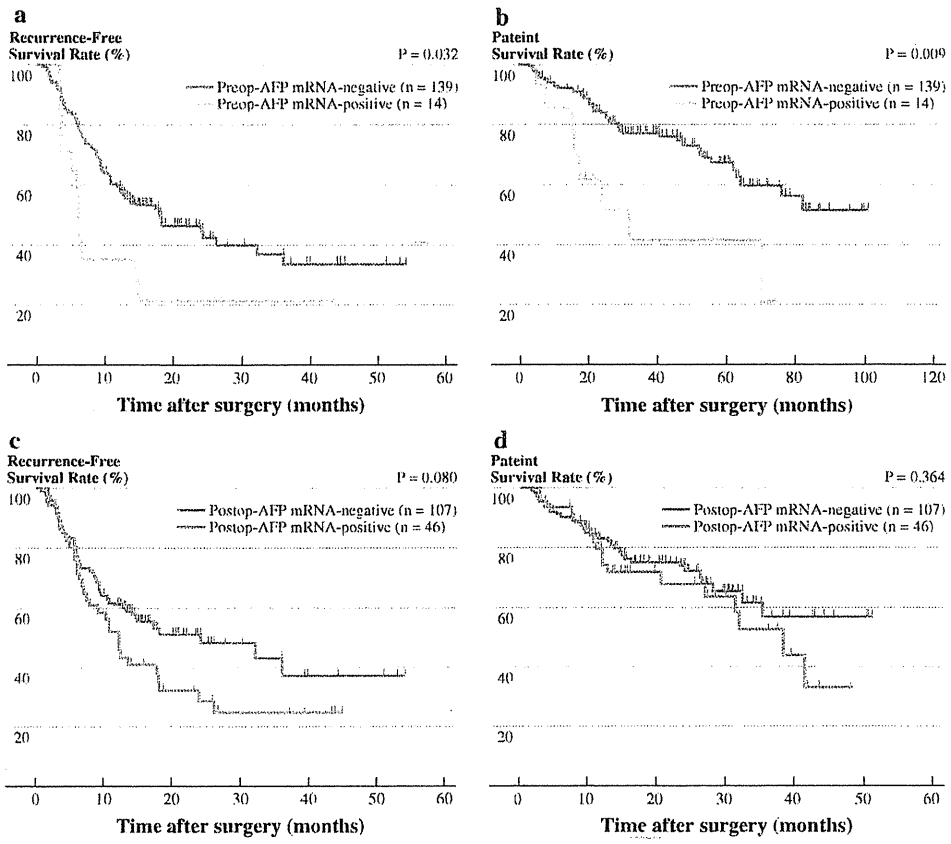

FIG. 2 Kaplan–Meier plot of recurrence-free and overall survival rates after liver resection according to pre- and postoperative AFP mRNA status. **a** The recurrence-free survival rate was worst for patients positive for preoperative AFP mRNA expression in peripheral blood ($P = 0.032$). **b** The overall survival rate was worst for patients positive for preoperative AFP mRNA expression in peripheral blood ($P = 0.009$). **c** Postoperative AFP mRNA-positive patients tended to have the worst recurrence-free survival rates ($P = 0.080$). **d** There was no difference in the overall survival rates between postoperative AFP mRNA-positive and mRNA-negative patients

the MR group and EX group, suggesting that the mech anism of multiple intrahepatic HCC recurrence could be associated with circulating tumor cells as in the EX group, which was compatible with the statement in our previous report.[20] Furthermore, preoperative AFP mRNA was not associated with HCC recurrence in the SR group, whereas it was strongly associated with HCC recurrence in the MR and EX groups. These result are quite reasonable because HCC recurrence in the EX group develops with circulating tumor cells that can be detected by measuring AFP mRNA in peripheral blood. Furthermore, as the result of MR group showed, it is possible that HCC recurrence results from circulating tumor cells detectable in peripheral blood homing residual liver tissue.

Multivariate analysis identified well-known risk factors, including tumor number, and microscopic vascular invasion as independent risk factors for HCC recurrence, which was compatible with other reports published previously, although tumor size was not an independent risk factor.[21] We applied TACE preoperatively to patients with tumors measuring >5 cm, and there was a strong correlation between TACE and tumor size ($P < 0.0001$). One reason why tumor size was not an independent risk factor for HCC recurrence in our series is that TACE was effective in reducing HCC recurrence postoperatively. AFP correlated with AFP mRNA expression in the peripheral blood. AFP is a well-known predictor of HCC recurrence. However, the presence of circulating tumor cells has a quite different meaning from the release of AFP from tumor cells. The association of these predictors can be interpreted to reflect the release of large amounts of AFP from advanced HCC and transmigration of HCC cells from the liver into the systemic circulation.

AFP mRNA positivity was defined using a cutoff level (relative ratio to GAPDH more than 1.0×10^{-8}) of AFP mRNA quantification in our studies. Although there may be considered potential increase of the risk of HCC recurrence associated with higher level of preoperative AFP mRNA, there was no relation between preoperative AFP mRNA level and these risk factors of tumor characteristics and HCC recurrence (Table 3).

The ability to predict HCC recurrence preoperatively is certainly clinically useful. Although there is solid evidence that preoperative intervention, such as TACE or radiofrequency ablation, results in suppression of tumor recurrence and improves prognosis, it is possible that the status of AFP mRNA would predict the efficacy of preoperative

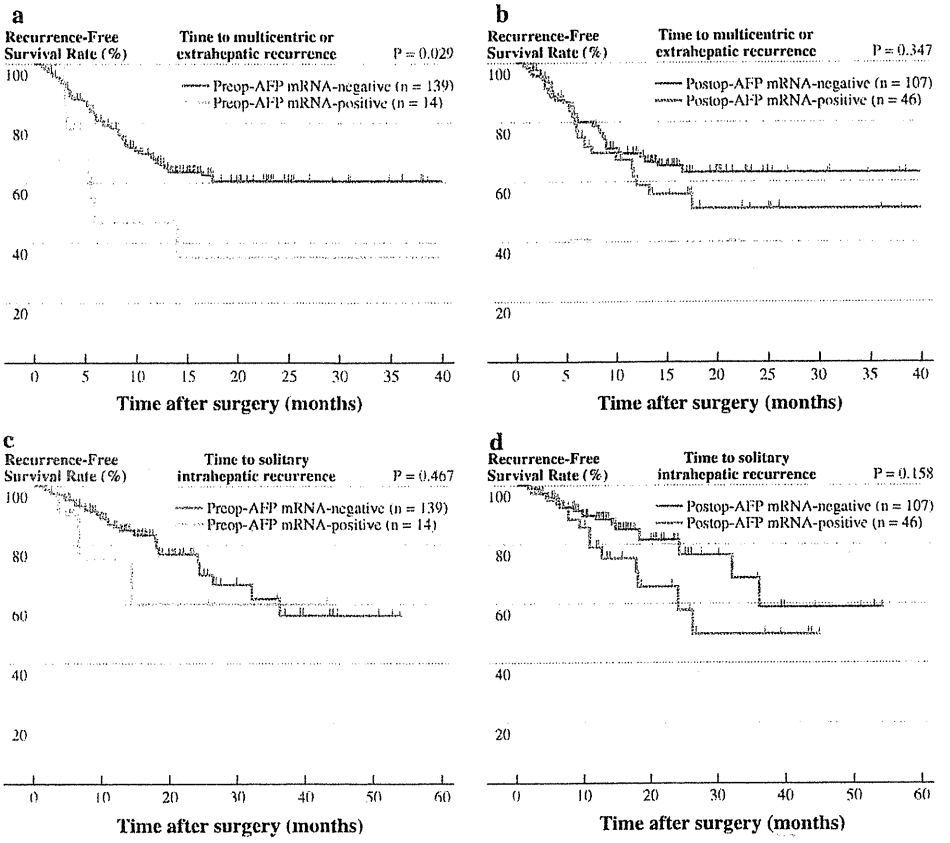

FIG. 3 Kaplan–Meier plot of recurrence-free survival rates after liver resection, censored by HCC recurrence in the SR group (="time to solitary intrahepatic recurrence") and MR/EX combination group (="time to multicentric or extrahepatic recurrence"). **a** MR/EX recurrence-free survival rate according to "time to multicentric or extrahepatic recurrence." HCC recurrence in the MR/EX group was censored. Patients were divided according to the status of preoperative AFP mRNA expression. Preoperative AFP mRNA-positive patients had the worst recurrence-free survival rate ($P = 0.029$). **b** Recurrence-free survival rates for the MR/EX group, divided according to the status of postoperative AFP mRNA according to "time to solitary intrahepatic recurrence." HCC recurrence in the SR group was censored. The status of postoperative AFP mRNA did not influence the recurrence-free survival rate. **c** SR recurrence-free survival curves. Patients were divided according to the status of preoperative AFP mRNA. HCC recurrence of the MR/EX group was censored. **d** SR recurrence-free curves, divided according to the status of preoperative AFP mRNA. HCC recurrence of the MR/EX group was censored. The status of AFP mRNA did not influence the recurrence-free survival rate

locoregional treatment in patients found preoperatively positive for AFP mRNA. Furthermore, the indication for hepatectomy in patients found preoperatively positive for AFP mRNA is an important issue, which requires comparative cohort study or a randomized, clinical trial.

One of the limitations of detecting AFP mRNA preoperatively to predict HCC recurrence is the low sensitivity of the RT-PCR used for AFP mRNA (as low as 12.9%) and relatively high specificity (78.6%). The low sensitivity of this method to detect circulating tumor cells could be due to the small sample of blood obtained (16 ml), which may not be adequate for detecting viable circulating tumor cells. Another possibility is that patients with very advanced HCC and macroscopic vascular invasion were excluded from this study to simplify the study design. An alternative approach would be to find a better biomarker to distinguish potent CTCs from dying CTCs, which may explain the high detectability of AFP mRNA postoperatively without association with HCC recurrence. Nonetheless, it is notable and quite important finding that the specificity of preoperative AFP mRNA to predict HCC recurrence was high, and that recurrence-free or overall survival was significantly worse according to the preoperative AFP mRNA status.

We evaluated bone marrow samples for detection of AFP mRNA in a previous study.[2] However, the expression of AFP mRNA in the bone marrow did not correlate with HCC recurrence; therefore, we did not evaluate bone marrow samples in the present study.

In conclusion, the results of the present study show that detection of AFP mRNA preoperatively in peripheral blood

is a useful predictor of multiple intrahepatic and extrahepatic HCC recurrence and that the expression of AFP mRNA does not predict de novo HCC.

CONFLICT OF INTEREST None.

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LIVER TRANSPLANTATION 16:33-37, 2010

ORIGINAL ARTICLE

Salvage Cystic Duct Anastomosis Using a Magnetic Compression Technique for Incomplete Bile Duct Reconstruction in Living Donor Liver Transplantation

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In living donor liver transplantation (LDLT), bile duct reconstruction is often technically demanding due to the frequently anomalous anatomy of the bile duct, as well as the high incidence of biliary complications. A bile duct branch may also be accidentally left without anastomosis at the time of LDLT and found to be obstructed after surgery. Surgical revision for such cases is sometimes not feasible because of the invasiveness of the procedure. We report a case in which a bile duct branch was intentionally left without anastomosis and was later successfully anastomosed to the cystic duct stump using a magnetic compression anastomosis (MCA) technique. A combination of the MCA technique and cystic duct anastomosis is life-saving in certain situations and should be considered as the treatment of choice. *Liver Transpl 16:33-37, 2010.*

Reconstruction of the bile duct in living donor liver transplantation (LDLT) is often complicated because of anatomical variations, especially in right lobe liver transplantation.[1] In addition, the high incidence of biliary complications affects the postoperative course.[2]

Bile duct strictures are the most commonly encountered biliary complications after liver transplantation.[3,4] Treatment for bile duct strictures includes nonsurgical methods such as endoscopic or percutaneous bile duct balloon dilatation and stent placement, surgical revision of the bile duct anastomosis, and retransplantation in the case of allograft failure. However, endoscopic or percutaneous treatments often fail with relapse of the stricture, whereas surgical revision and retransplantation are more invasive and are associated with much morbidity.

It is possible for occlusion of the bile duct anastomosis to result as an advanced condition of a stricture[5] or to result from a bile duct branch accidentally or intentionally left without anastomosis in partial liver transplantation. In these cases, an endoscopic or percutaneous approach would not be effective, and surgical revision would be necessary for reconstruction, although it is invasive and is associated with much morbidity. The magnetic compression anastomotic technique is a simple, noninvasive, and rather effective method for recanalizing a bile duct obstruction, and we have previously reported its application in liver transplant recipients.[6]

Duct-to-duct anastomosis has now become the standard biliary reconstruction method because of its advantages over hepaticojejunostomy in both deceased

Abbreviations: B#, branch of segment #; CBD, common bile duct; ESLD, end-stage liver disease; LDLT, living donor liver transplantation; MCA, magnetic compression anastomosis; PTCD, percutaneous transhepatic cholangiodrainage.

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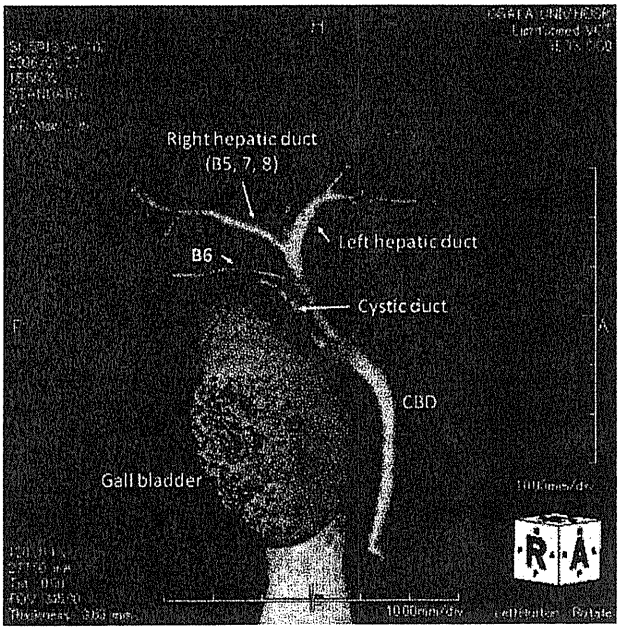

Figure 1. Three-dimensional reconstruction view of the drip infusion cholangiography computed tomography scan. The donor B6 bile duct rose from the common hepatic duct separately from the right hepatic duct (B5, B7, and B8).

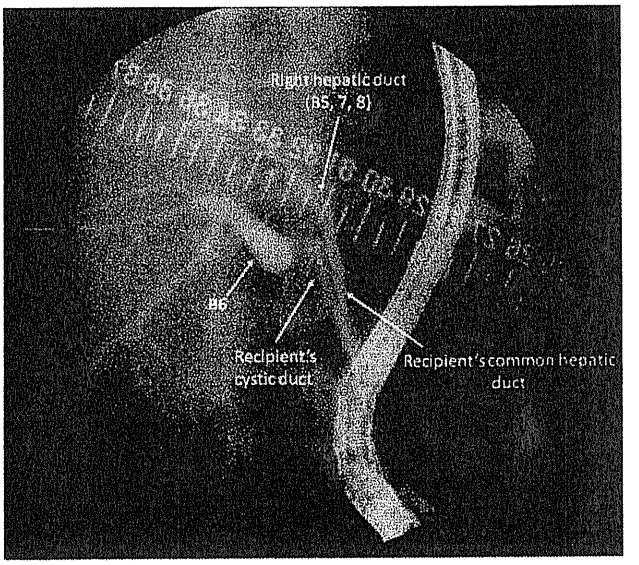

Figure 2. Cholangiography in the recipient before the magnetic compression anastomosis. Simultaneous endoscopic retrograde cholangiography combined with B6 tube cholangiography showed dilatation of the B6 bile duct and the stump of the cystic duct close to it. Duct-to-duct anastomosis of the right hepatic duct did not result in a stricture.

donor liver transplantation and LDLT.[7,8] However, duct-to-duct reconstruction to the recipient cystic duct in LDLT is a relatively new and unique concept; it was described first by Suh et al.[9] and then by Asonuma et al.[10] and has been shown to be safe and useful.

We report herein a case in which a bile duct branch was intentionally left without anastomosis and was later successfully anastomosed to the cystic duct stump with a magnetic compression technique.[6,11] The successful outcome in the present case is believed to have resulted from a combination of the magnetic compression anastomosis (MCA) technique and cystic duct anastomosis.

CASE REPORT

A 53-year-old male patient with ESLD secondary to hepatitis B virus was referred to our hospital. A preoperative evaluation revealed a small hepatocellular carcinoma (single, 10 mm) and no abnormalities of the cardiopulmonary systems. Renal function was impaired, with the serum creatinine level at 1.5 mg/dL and creatinine clearance at only 24.9 mL/minute.

His brother volunteered to donate his liver, and after extensive donor evaluations, including computed tomography volumetry of the liver, it was determined that only the right lobe could be donated, even though there was a small bile duct branch of segment 6 (B6) arising separately from the right hepatic duct (Fig. 1).

The transplant surgery was quite complicated because of the presence of portal hypertension with massive ascites, extensive coagulopathy, and severe fibrosis around the cirrhotic liver. Hepatectomy was performed as usual by the piggyback technique, and the right lobe graft was anastomosed without difficulty. Bile duct reconstruction between the donor right bile duct (B5, B7, and B8) and recipient common hepatic duct was performed with 6-0 absorbable monofilament interrupted sutures with an external tube across the anastomosis. At that time, we found that the jejunum was extensively edematous. Furthermore, the B6 bile duct was so small that we decided not to proceed with anastomosis of this bile duct, and instead we placed a 5-Fr external biliary tube. We initially planned a second-look operation of the hepaticojejunal anastomosis when the patient stabilized postoperatively.

A septic event with methicillin-resistant *Staphylococcus aureus* pneumonia necessitated reintubation and tracheotomy during the first week after LDLT. The postoperative initial graft function gradually recovered during the first 6 months without any surgical morbidity or acute cellular rejection until he suddenly developed acute heart failure of an undetermined cause with an ejection fraction of 20% at 6 months after LDLT. Subsequently, his renal function also deteriorated, and this necessitated continuous venovenous hemodialysis for 16 days. The heart failure gradually improved, with the ejection fraction recovering to 45% at post-operative month 12, whereas the renal function never recovered, with the serum creatinine level remaining around 3 mg/dL despite maintenance of normal urine output.

Surgical relaparotomy under general anesthesia for anastomosis of the B6 bile duct was considered to be contraindicated at this time point because of the multiple-organ failure. One of the solutions under these circumstances is to clamp the B6 drainage tube or inject ethanol to destroy the bile duct epithelium; how-

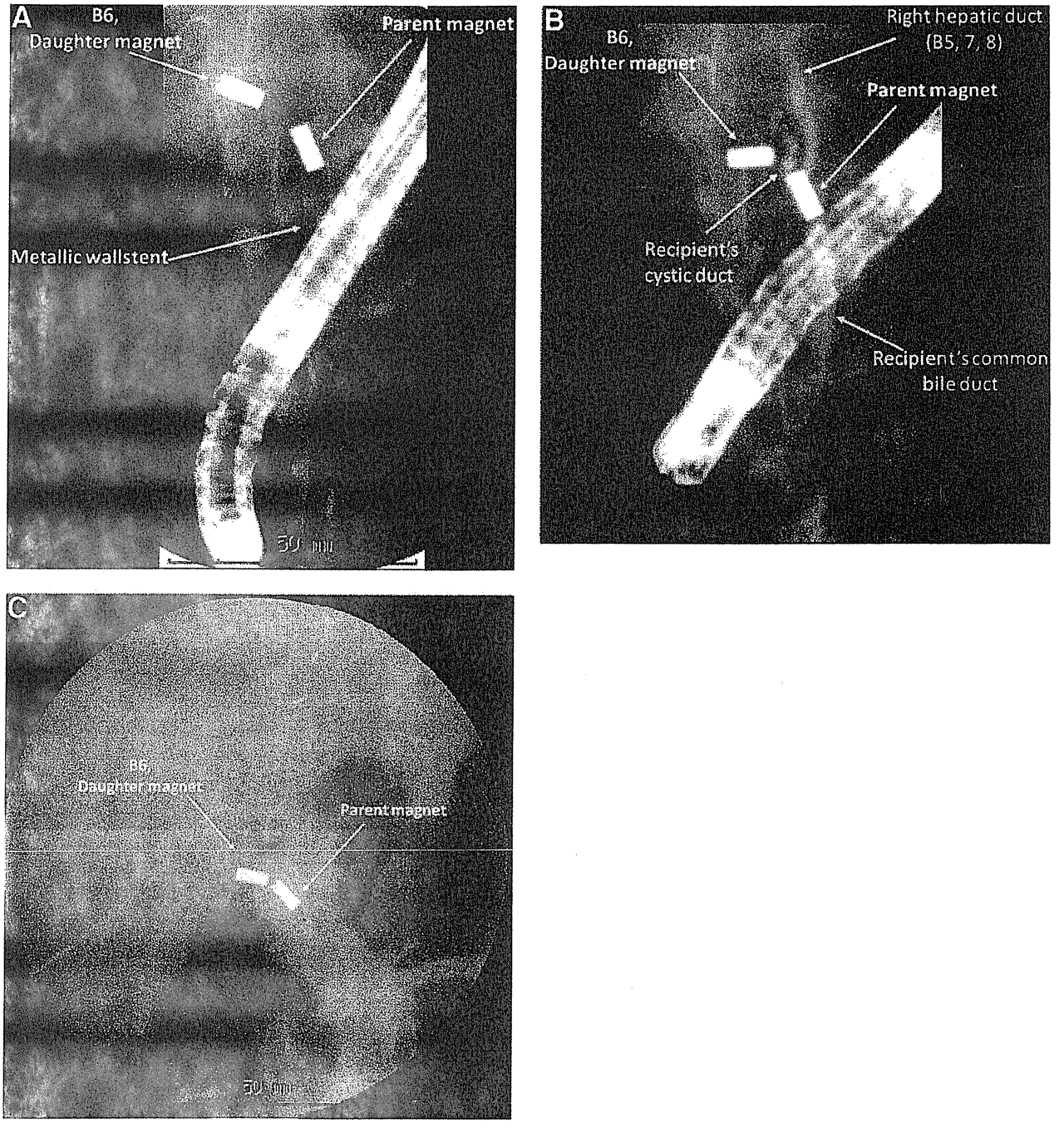

Figure 3. Magnetic compression anastomosis between the B6 bile duct and recipient's cystic duct. (A) The parent magnet was brought to the stump of the cystic duct via the common bile duct with the metallic Wallstent technique described previously. The daughter magnet was brought to the B6 bile duct via the percutaneous transhepatic cholangiodrainage route. (B) The patency of the common bile duct was confirmed by the injection of 60% urografin. (C) The parent and daughter magnets came close together by the end of this procedure.

ever, these were not recommended in this case because the area of B6 was relatively large and the bile drainage through the B6 bile duct drain was about 200 mL. Magnetic resonance imaging showed sufficient dilatation of the B6 bile duct for a percutaneous transhepatic cholangiodrainage (PTCD) tube to be inserted and placed (Fig. 2). Fortunately, the cystic duct stump was relatively close to the stump of the B6 bile duct.

The PTCD tube was inserted and sized up to 16 Fr, and MCA was performed successfully at POM 14 with a metallic Wallstent technique described previously.[6] A daughter magnet was first delivered to the end of B6.

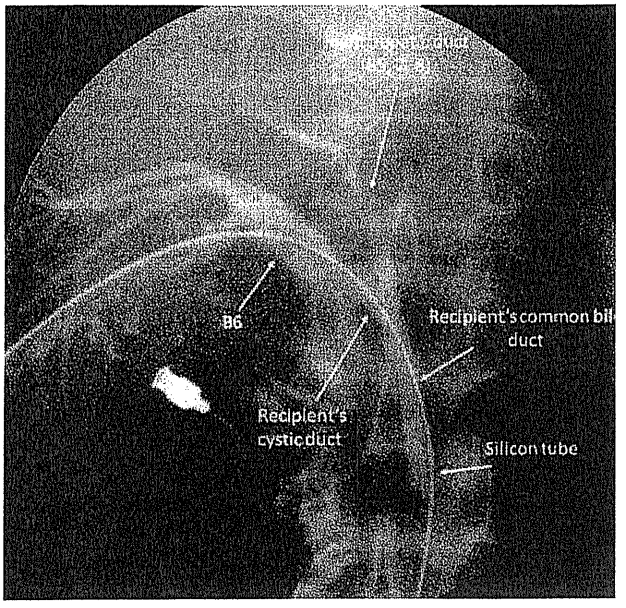

Figure 4. Cholangiography after the magnetic compression anastomosis. Both magnets were removed via the percutaneous transhepatic cholangiodrainage route. A 14-Fr silicon tube was inserted and placed.

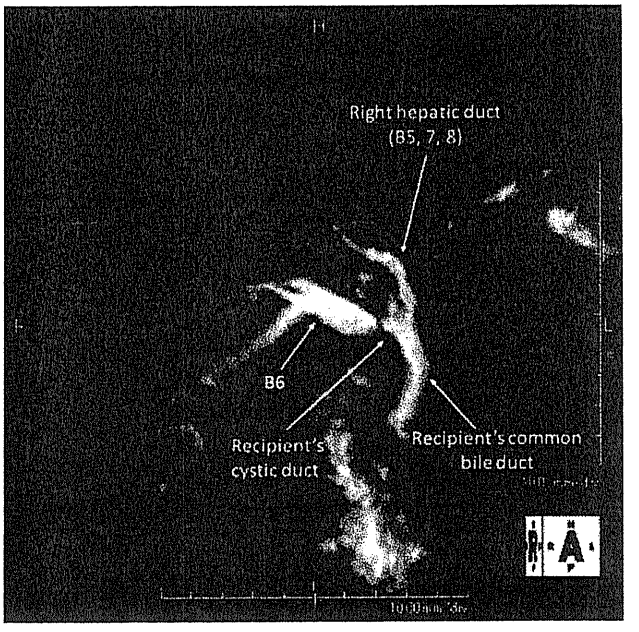

Figure 5. Magnetic resonance cholangiopancreatography image 6 months after the removal of the percutaneous transhepatic cholangiodrainage tube. There was narrowing of the magnetic compression anastomosis segment. The original anastomosis of the right hepatic duct was secure.

After endoscopic sphincterotomy, a metallic Wallstent [Wallstent RX biliary metal stent (8 mm in diameter and 60 mm long). Boston Scientific Japan K.K., Tokyo, Japan] was inserted into the cystic duct stump via the papilla of Vater and common bile duct. Then, the parent magnet was delivered safely into the cystic duct stump without interference from the common bile duct (Fig. 3A); this was confirmed by fluoroscopy (Fig. 3B). The Wallstent was removed immediately after the placement of the magnets, and the 2 magnets came close together (Fig. 3C) by the end of the procedure. There was no deterioration of the liver function after the placement of the magnets. On day 63 after the MCA procedure, both the parent and daughter magnets were removed, and a safety PTCD tube was placed across the B6 bile duct and cystic duct (Fig. 4). At 12 months after the MCA, the B6 biliary tube was removed after cholangiographic confirmation of the absence of bile leakage from the biliary system. The PTCD tube was finally removed at 18 months after the MCA procedure. Magnetic resonance imaging performed 6 months after the PTCD removal showed no bile leakage and homogeneous parenchyma in the entire graft, including segment 6, although the MCA region could not be visualized well by magnetic resonance cholangiography (Fig. 5). A hepatobiliary iminodiacetic acid scan showed normal bile drainage from B6 to the common bile duct, and this suggested normal functioning of the MCA site despite the lack of visualization of the tube between them on magnetic resonance cholangiography. The patient is now under routine follow-up at the outpatient clinic without any complaints or evidence of biliary infection and with normal serum aminotransferase, gamma glutamyl transferase, and alkaline phosphatase levels 10 months after the PTCD removal.

DISCUSSION

The clinical course of this case was uncommon because of the anomaly of the B6 bile duct and the unexpected deterioration of the heart and renal function after LDLT in the patient; however, the problem of the bile duct anastomosis of B6 was successfully resolved by a nonsurgical procedure using the MCA method. This method could be applied as one of the alternatives for performing multiple biliary reconstructions or rescuing a biliary branch that has been unexpectedly ligated during donor hepatectomy. Usage of the cystic duct has the advantage of avoidance of interference by bile flow from the rest of the grafted liver. Although it is sometimes difficult to deliver a magnet via the papilla of Vater for this MCA technique, we applied a metallic stent technique described previously[6] and successfully delivered the magnet without difficulty.

In conclusion, this case illustrates an alternative method for biliary reconstruction. A small bile duct branch in right lobe grafts can be anastomosed in a second-look fashion by the MCA technique, and this should be considered one of the options for bile duct reconstruction.

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Evaluation of a New Immunoassay for Therapeutic Drug Monitoring of Tacrolimus in Adult Liver Transplant Recipients

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Therapeutic drug monitoring is necessary when using tacrolimus (FK) due to the associated side effects. The aim of this study was to compare the chemiluminescent assay (CMIA) system with the previously established Abbott IMx Tacrolimus II microparticle enzyme immunoassay (MEIA) in liver transplant recipients and evaluate its accuracy. Between March and June 2008, all blood samples from the liver transplant recipients at the hospital were tested for FK trough level using 2 different methods, CMIA and MEIA. The posttransplant time, hematocrit, and other clinical parameters during the study period were recorded. FK trough level was analyzed in 398 samples from 57 liver transplant recipients by CMIA and MEIA. The correlation in FK level between the 2 methods was excellent ($r^2 = 0.941$). However, the FK level was underestimated in MEIA by more than 23% in samples with an FK level of less than 3.5 ng/mL and by 6.8% in those with an FK level between 3.5 and 5 ng/mL. CMIA is superior to MEIA in measuring low FK level, allowing the FK level to be maintained at less than 5 ng/mL in selected liver transplant recipients. The effects of maintaining low levels of FK should be evaluated in liver transplant recipients.

Keywords: *tacrolimus; liver transplantation; therapeutic drug monitoring; immunoassay*

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Tacrolimus (FK) is a potent immunosuppressant known as a calcineurin inhibitor and a key drug used in organ transplantation. FK is effective in preventing rejection and maintaining organ function in liver transplantation, but its side effects, such as nephrotoxicity,[1] diabetes mellitus,[2] hypertension, and infection, are life threatening in posttransplant management.[3] Therefore, therapeutic drug monitoring is necessary for transplant recipients to secure a better posttransplant course. Reduced exposure to elevated blood concentrations of tacrolimus provides adequate immunosuppression and improved renal function in kidney transplantation.[4] In liver transplant recipients, tacrolimus concentration should be minimized, especially in stable recipients after transplantation.

The IMx Tacrolimus II microparticle enzyme immunoassay (MEIA; Abbott Laboratories, Abbott Park, Illinois) is a standard system for monitoring tacrolimus blood concentrations.[5] However, the reported limit of quantification (LOQ) in blood concentration of tacrolimus using MEIA is 4.1 ng/mL,[6] which may be high and harmful in stable liver transplant recipients. A trough level of tacrolimus around 3 to 4 ng/mL seems adequate and could be reduced to even perhaps around 1 ng/mL.[6] A once-a-day tacrolimus dose would require a target level as low as around 1 ng/mL, although the MEIA system does not support such low levels.

The newly developed ARCHITECT tacrolimus assay using the automated chemiluminescent assay (CMIA; Abbott Laboratories) for quantitative measurement of tacrolimus on the ARCHITECT i2000SR platform is highly sensitive and provides precise

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measurement. It also allows the management of low-target concentrations in liver transplant recipients.

To our knowledge, there are no reports that compare MEIA and CMIA in the assessment of tacrolimus in liver transplant recipients. The present study was designed to compare these 2 methods in liver transplant recipients and determine the efficacy of the ARCHITECT tacrolimus assay in clinical settings.

PATIENTS AND METHODS

Abbott IMx Tacrolimus II Microparticle Enzyme Immunoassay

In this immunoassay, whole-blood sample is mixed with the microparticle reagent, followed by incubation, in which tacrolimus in the sample binds to the antibody binding sites on the microparticles. An aliquot of the mixture is then transferred to a glass-fiber filter, and tacrolimus-alkaline phosphatase conjugate is added to the filter. Conjugates that do not bind to the remaining antibody binding sites on the microparticles are removed by washing. Then, the substrate reagent is added to the filter and the rate of appearance of the fluorescence product is measured by front surface fluorescence measurements. The rate is inversely proportional to the amount of the analyte present in the patient sample or standard.

Automated CMIA for the Quantitative Determination of Tacrolimus on the ARCHITECT i2000SR Platform

The CMIA method is based on the enzyme immunoassay (EIA) principle. In contrast to the EIA, where the antigen-antibody complexes are detected by enzyme-labeled conjugate, the CMIA uses an acridinium-labeled conjugate as the detection system. The LOQ of blood concentration of tacrolimus using CMIA is as low as 0.8 ng/mL.[6]

Comparison of MEIA and CMIA

During the study period from March to June 2008, peripheral blood samples from 57 liver transplant recipients were analyzed by both techniques. Among 57 recipients, 44 patients underwent living donor liver transplantation, and 13 received deceased donor liver transplantation.

All recipients were taking tacrolimus (Prograf) every 12 hours, and blood samples were obtained at trough level. Each blood sample was divided into 2 portions and each used immediately for 1 assay. The immunosuppressive protocol consisted of tacrolimus and steroid with or without mycophenolate mofetil (MMF) or tacrolimus and anti-CD25 monoclonal antibody with MMF. Tacrolimus was started at a dose of 0.03 mg/kg and titrated 8 to 12 ng/mL as trough level during the first 3 weeks after transplant, then reduced to 6 to 8 ng/mL in the first 3 months and 4 to 6 ng/mL thereafter.

The ARCHITECT ratio (AR) was defined as [C(ARCHITECT) – C(IMx)]/C(ARCHITECT), where C(ARCHITECT) is FK trough concentration in the CMIA method, and C(IMx) is that in the MEIA method. Samples were also divided according to hematocrit into <25%, 25% to 45%, and over 45%.

Statistical Analysis

Results are expressed as mean ± standard deviation or median. Statistical examination of the correlations was based on the Pearson product-moment correlation. A P value less than .05 was considered statistically significant.

RESULTS

Samples from 57 liver transplant recipients (43 living donor liver transplantation and 14 deceased donor liver transplantation), including 3 patients who received liver transplantation during the study period, were analyzed (Table I). Time after liver transplantation ranged from 0 to 7.7 years (3.5 ± 2.2 years). The primary diagnoses of these 57 patients are listed in Table I. The total number of blood samples analyzed by the 2 methods was 398, including 35 samples within 3 weeks after liver transplantation, 57 samples between 3 weeks and 3 months, 93 samples between 3 months and 1 year, and 213 samples over 1 year. FK trough level was 11.9 ± 3.1 (MEIA), 11.5 ± 2.8 (CMIA) within 3 weeks; 11.3 ± 3.2 (MEIA), 10.8 ± 2.8 (CMIA) between 3 weeks and 3 months; 8.2 ± 4.5 (MEIA), 8.0 ± 4.1 (CMIA) between 3 months and 1 year; and 6.4 ± 3.3 (MEIA), 6.5 ± 2.9 (CMIA) over 1 year.

FK trough level ranged from 0.2 to 23.0 ng/mL in the MEIA method and from 1.4 to 21.9 in the CMIA method. The FK level in all samples was above the LOQ in the CMIA method (0.8 ng/mL), whereas 13.4% of all samples were below the LOQ (4.1 ng/mL) in the MEIA method. Samples with hematocrit less than 25% accounted for 4.0% (n = 16), whereas