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Alteration of Bile Acid Transporter Expression in Patients with Early Cholestasis Following Living Donor Liver Transplantation

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Background : Intrahepatic cholestasis can occur early after living donor liver transplantation (LDLT). We investigated the changes in the expressions of the bile acid transporters and the liver histology in the patients who suffered with early cholestasis (EC) following LDLT. **Methods :** The histological differences between 15 graft livers with EC after LDLT and 5 graft livers with biliary stricture following LDLT were evaluated. The hepatic mRNA levels of the bile canaliculi transporters (BSEP, MRP2, MRP3, MDR1, MDR3, NTCP) in 40 (20 graft livers, 20 matched donor livers) liver biopsy tissues were analyzed by performing real-time reverse-transcription polymerase chain reaction (RT-PCR). **Results :** Microscopic examination revealed hepatocellular and/or bile canalicular cholestasis around acinar zone 3 in the livers of the patients with EC. In the livers with biliary stricture, the cholestasis was dominantly observed in the hepatocytic cytoplasm and in the bile ductules around the portal area rather than around acinar zone 3. The BSEP and MRP2 mRNA levels in the EC livers were significantly reduced by 44% and 23%, respectively (p=0.000), compared to the matched donor livers. The levels of MDR3 and NTCP mRNA in the EC livers increased by 738% (p=0.000) and 281% (p<0.01), respectively. The change of the expressions of the bile acid transporters in the patients with biliary stricture was less significant than that in the EC group. **Conclusions :** These results suggest that the altered expressions of the bile acid transporters may play a role in the pathogenesis of EC following LDLT.

Key Words : Cholestasis; Liver transplantation; Bile acid transporter

Mild cholestasis is common following liver transplantation, but severe cholestasis can also occur. Severe cholestasis is defined as an increase in the serum bilirubin level to greater than 100 μmol/L.[1] Severe intrahepatic cholestasis can be divided into the early and late phase cholestasis depending on the time of occurrence because these 2 phases usually have distinct etiologies. Early cholestasis is generally defined as occurring within 6 months of transplantation, while late cholestasis is generally defined as occurring after 6 months or more.[1] The reported causes of early severe cholestasis following liver transplantation (LT) are ischemia-reperfusion injury, initial graft dysfunctions, infections, biliary obstruction, a small graft size, drug toxicity and acute cellular rejection.[2,3] Yet researchers have found that early cholestasis could develop approximately 2 to 3 weeks following LT without any definitive causes of the cholestasis.

Bile acid transport under physiologic conditions is mediated by bile acid transporters (BSEP, MRP2, MPR3, NTCP, MDR1, MDR3) that are localized to the sinusoidal and canalicular membranes of hepatocytes and cholangiocytes.[4,5] In most of the cholestatic disorders, transporter alterations are the consequence rather than cause of cholestasis. Such transporter alterations generally represent an attempt to adapt to the accumulating biliary constituents that are observed in patients with cholestasis and to protect the hepatocytes from the intracellular accumulation of toxic bile acids.[6,7] It is very important in the pathophysiology of early cholestasis following living donor liver transplantation (LDLT) to determine if the adaptive alterations of these bile acid transporter systems function during the early cholestasis following a LDLT.

Therefore, this study was conducted to compare the histological characteristics of the early cholestasis following LDLT, and this occured without any known specific causes, with those of

the cholestasis that was the result of biliary stricture. Furthermore, we investigated the changes of 6 major bile acid transport systems in the percutaneous liver biopsy tissues of the patients with early cholestasis following LDLT, as compared to the matched donor liver, and we used real-time reverse-transcription polymerase chain reaction (RT-PCR) in order to gain insight into the role that transporter regulation plays in the early cholestasis that develops following LDLT.

MATERIALS AND METHODS

Patients

Between June 1999 and December 2003, a total of 105 adult LDLT were performed at our liver transplantation center. All the LDLT recipients received the right-lobe from an adult living donor. The 1 year and 3 year survival rate of the recipients was 91% and 85%, respectively. There were no cases of mortality for any of the living donors included in this study. The study group included 15 consecutive patients who developed early cholestasis, for which the cause was unknown, within 6 months of the LDLT. The causes of early cholestasis, including ischemia/reperfusion injury, bacterial infection, acute cellular rejection and cytomegalovirus infection, were ruled out by liver biopsy, the laboratory data, the graft/recipient body weight and the surgical records. The second group was comprised of 5 consecutive patients who developed cholestasis following LDLT due to biliary stricture. The clinical characteristics of the matched donor and transplant recipients at the time of the percutaneous liver biopsy are summarized in Table 1. The control group was comprised of the 20 matched living donors of the cholestasis patients. Informed consent was obtained from each patient prior to surgery and tissue utilization, and all the sample collections were performed with following the hospital guidelines for tissue banking. Cyclosporine (CsA)-based immunosuppression (cyclosporine, mycophenoleate mofetil, hydrocortisone) was used for all the 20 evaluated patients. The target trough level of CsA was approximately 200-250 ng/mL, as determined by monoclonal specific immunoassays (TDx FPIA, radioimmunoassay, Cyclo-Trac) during first month. This study's protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Liver tissues

Fifteen liver tissue specimens were obtained during diagnostic liver biopsies, which were conducted using an 18-gauge biopsy gun during the early post-transplantation period following LDLT. In addition, 5 liver tissues were obtained from 5 patients with cholestasis, and the cholestasis occurred due to biliary stricture following LDLT. Furthermore, 20 fresh normal liver tissue samples were obtained from the right lobes of 20 matched living donors during the transplantation operation.

One portion of each liver biopsy specimen was immediately snap-frozen and stored in liquid nitrogen until the total RNA was extracted. The remaining material was fixed with 10% formalin solution and then it was evaluated by routine histology and immunohistochemistry.

The mRNA expression of biliary canalicular transporter by real-time PCR

The total RNA was extracted from the frozen liver tissue specimens using TriZol reagent and following the manufacturer's protocols (Molecular Research Center, Inc. Cincinnati, OH, USA). The corresponding cDNA was then synthesized using a Thermoscript kit (Invitrogen, Carlsbad, CA, USA) and the RNA (0.1 μg) obtained from the liver samples. One microliter of the cDNA was then used as the template for quantitative RT-PCR. The primer mix, the templates and 2× QuantiTech SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) were mixed and then diluted to a final volume of 10 μL. PCR was then conducted by subjecting this mixture to 40 cycles at 95℃ for 30 s, 57℃ for 30 s and 72℃ for 30 s with using a Rotor-gene real-time

Table 1. Clinical characteristics of the transplant recipients at the time of the percutaneous liver biopsy

	Early cholestasis group	Biliary stricture	Control donor liver	p-value
No	15	4	10	
Mean age (range, years)	47 (37-57)	44 (42-53)	36 (30-39)	
Mean post-LT interval (range, days)	25 (21-35)	20 (19-24)		
Total bilirubin (mg/dL) AQ	6.6±1.1	14.9±2.8	1.5±0.7	p<0.05
AST (IU/L)	182±28	40±1	23±2.8	
ALT (IU/L)	392±72	103±31	24±3.8	
Alk-p (IU/L)	483±112	170±49	180±29	
r-GTP (IU/L)	12±18	33±14	45±7	
Underlying diseases				
Liver cirrhosis	11	3		
HCC	4	1		

AST, aspartate aminotransferase; ALT, alanine aminotransferase; Alk-p, alkaline phosphatase; r-GTP, gamma glutamyl transpeptidase; HCC, hepatocellular carcinoma.

PCR machine (Corbett Research Inc., Sydney, Australia). The specific primers used to amplify the Na^+/taurocholate cotransporter (NTCP), the bile salt export pump (BSEP), the multidrug export pump (MDR1), canalicular phospholipid flippase (MDR3), the canalicular conjugate export pump (MRP2) and the basolateral MRP homologue (MRP3) are all shown in Table 2. All the experiments were performed at least twice.

Selecting a suitable normalization factor is very important when conducting quantitative RT-PCR. In a previous study, we performed quantitative RT-PCR with using 13 housekeeping genes and 67 liver tissue samples, and we then analyzed the results with the geNorm program.[8,9] The results of that experiment revealed that the bilane synthase (HMBS) and ubiquitin C (UBC) genes are the best normalization factors in the liver.[9] Therefore, we used the geometric mean of the two normalization factors as the normalization factor in this study to correct for any differences in the efficacy of RNA extraction and the RNA quality.

Statistical analysis

The individual transporter mRNA expression levels were reported as values relative to the Ubiquitin C mRNA level. All the values shown represent the mean±the standard error. The differences in the mRNA expression among the patient groups were analyzed by a Mann-Whitney rank sum test (2-tailed) with using SPSS for Windows, version 10.0 (SPSS Inc., Chicago, IL, USA). A p-value <0.05 was considered to be significant for all the analyses.

RESULTS

The histological characteristics

In the early cholestasis group (n=15) cholestasis was primarily observed in the acinar zone 3 following LDLT, and this was often associated with the formation of an intracanalicular bile plug and sinusoidal infiltration of pigment-laden macrophages without ballooning degeneration around acinar zone 3. However, this was not found in the periportal area (Fig. 1A-C)

On comparison, the cholestasis of the biliary stricture group (n=5) was primarily found around the portal area and it was often associated with bile plugs in the bile ductules and with lymphocytic or neurtophilic infiltration. In addition, there was intracytoplasmic cholestasis of the hepatocytes observed around the acinar zone 3 (Fig. 1D, E). Sinusoidal infiltration by pigment-laden macrophages was not observed.

Table 2. Specific primers used for real-time PCR

Genes	Forward primer	Reverse primer
BSEP	5′-GGAACCAGTGTTGTTTGCCT-3′	5′-AAAATCATGCAGCTGAGCCT-3′
NTCP	5′-CCCAAGAAGCCTCACCTATC-3′	5′-TTGGGTCACAAAACTTGGAA-3′
MDR1	5′-GGAACTCAGCTCTCTGGTGG-3′	5′-CTTCTCTGGCTTTGTCCAGG-3′
MDR3	5′-AAGCAAGAGGCTGAGATGGA-3′	5′-TTTTTGTTTGCTGCTGATGC-3′
MRP2	5′-CTGGTTGGGAACCTGACTGT-3′	5′-CAACAGCCACAATGTTGGTC-3′
MRP3	5′-GGAGACTGACAACCTCATCC-3′	5′-ATTCTGCGGACATATTTGGT-3′
Ubiquitin	5′-CCTGGTGCTCCGTCTTAGAG-3′	5′-TTTCCCAGCAAAGATCAACC-3′
HMBS	5′-GGCAATGCGGCTGCAA-3′	5′-GGGTACCCACGCGAATCAC-3′

BSEP, bile salt export pump; NTCP, Na^+/taurocholate cotransporter; HMBS, hydromethyl-bilane synthase.

Table 3. mRNA expression of the hepatobiliary transporters in the liver tissues

Gene	Control donor (n=10)		Early cholestasis (n=15)		Biliary stricture (n=5)	
	Copy no	%	Copy no	%	Copy no	%
BSEP	75.6±6.8†	(100)	33.2±3.5†	(44±5)	60.8±12.4	(80±16)
MDR1	23.6±4.3	(100)	20±2.2	(85±9)	31.4±7.9	(133±33)
MDR3	22.2±2.5†,‡	(100)	163.8±25.7†	(738±116)	93.7±29.4‡	(423±132)
MRP2	135±6.8†,‡	(100)	30.8±4.1†	(23±3)	71.1±16.9‡	(53±13)
MRP3	137.1±5.7	(100)	119.3±15.5	(87±11)	176.8±16.9	(129±12)
NTCP	482.8±42.9*,‡	(100)	1,355.6±195.2*	(281±40)	1117.3±296.9‡	(231±61)

Transporter mRNA levels were normalized to those of Ubiquitin C and HMBS to account for differences in the efficacy of the RNA extraction and RNA quality. The values are expressed as the real copy number (×10,000) (mean±SEM); *, p<0.01; †, p=0.000, ‡, p<0.05. The abundance of each type of mRNA is expressed as a percentage of the control donor mRNA level in each specimen.

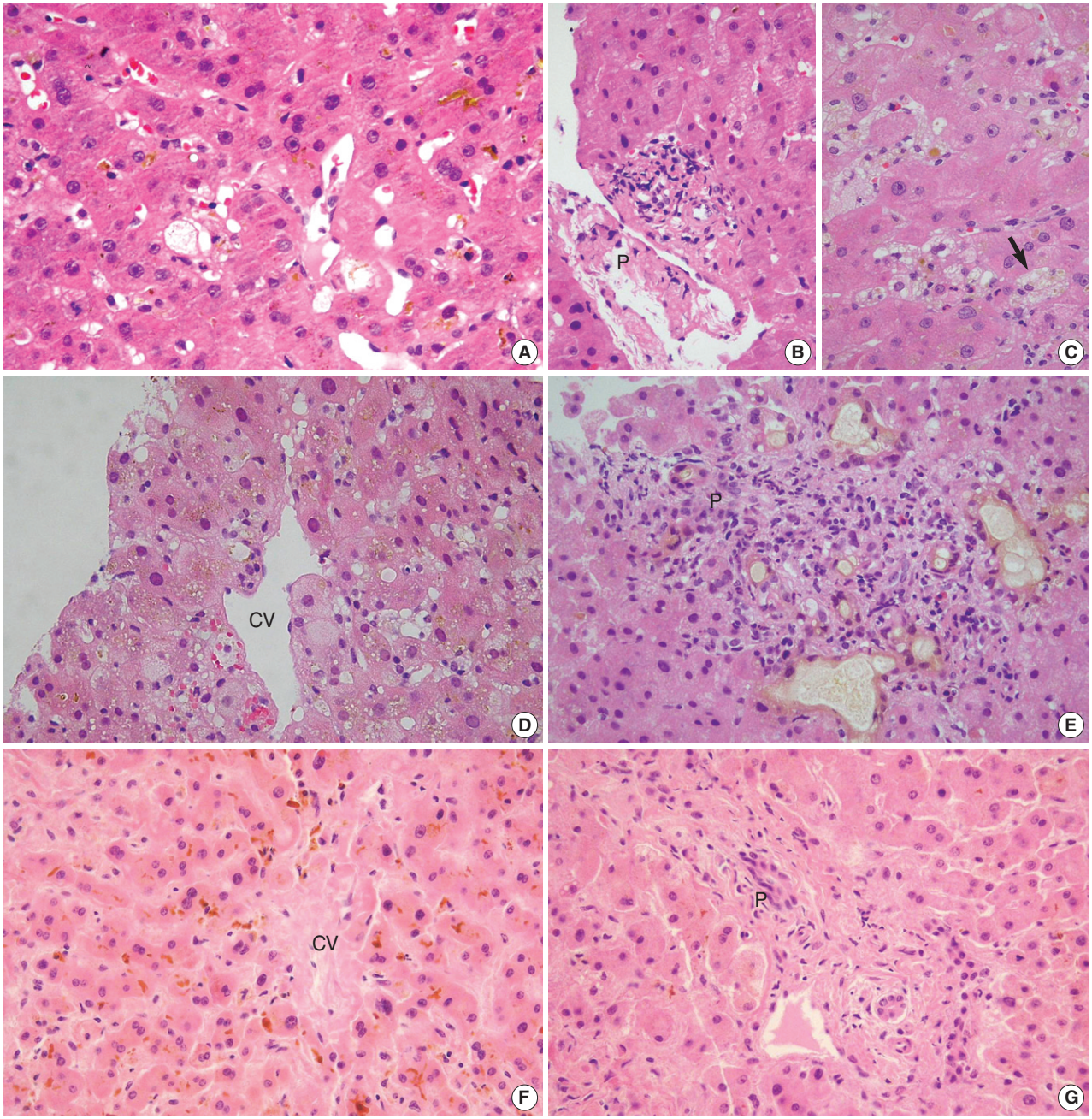

Fig. 1. Histological features of the early cholestasis group, biliary group and functional cholestasis group (H&E stain, 400×). The early cholestasis group shows central intracanalicular cholestasis (A), a well preserved portal area (B), and sinusoidal infiltration of pigment laden histiocytes (arrow) (C). The biliary group shows mild intracytoplasmic cholestasis around the acinar zone 3 (D), cholestasis in the portal tract associated with bile plugs in the bile ductules and lymphocytic or neutrophilic infiltration (E). The functional cholestasis group has central intracanalicular cholestasis and hepatocytic degeneration (F) and a well preserved portal area (G).
cv, acinar zone 3; p, portal tract.

The mRNA expression profile of the biliary canalicular transporters

The canalicular BSEP mRNA levels were reduced in the early cholestasis group as compared to the control group (44±5% vs. 100%, respectively $p<0.05$), yet the MRP3 mRNA levels were similar in all the groups. It is interesting that the MRP2 mRNA levels were down-regulated in the early cholestasis group (23±3%, $p=0.000$) and in the biliary stricture group (53±13%, $p< 0.05$). Conversely, the NTCP mRNA levels were up-

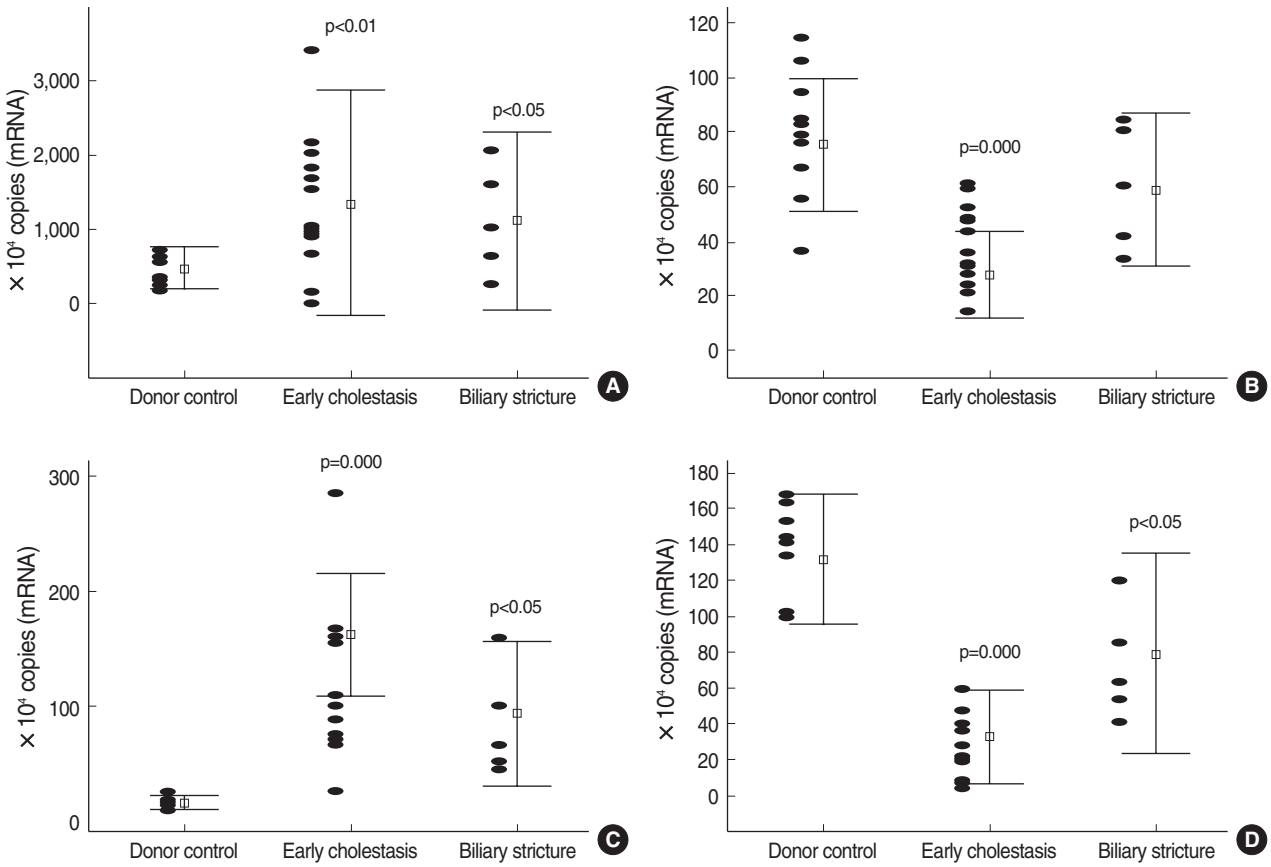

Fig. 2. BSEP, MDR3, MPR2 and NTCP mRNA expression in liver tissues. Following isolation of total RNA from percutaneous liver biopsy samples, cDNA synthesis and real-time RT-PCR were performed as described in the Material and Methods. Transporter mRNA copy numbers were then normalized to those of ubiquitin to correct for differences in the efficiency of RNA extraction and RNA quality. The values are expressed as the real copy number (×10,000). The box and the error bar represent the mean±2SD. Each data point represents the mean of the values of at least 2 independent real-time RT-PCR analyses per sample. (A) When compared with the control donor liver (n=10), the BSEP mRNA levels were reduced by 44% in patients with early cholestasis (p=0.000), while no significant changes were observed in the biliary stricture group (n=5). (B) When compared with the controls, the MDR3 mRNA levels were 738% higher in patients with early cholestasis (n=15) (p=0.000), and 423% higher in patients with biliary stricture (n=4) (p<0.05). (C) MRP2 mRNA levels were reduced by 23% in patients with early cholestasis (n=15) (p=0.000), and by 53% in biliary stricture (n=4) (p<0.05). (D) When compared with the controls (n=10), the NTCP mRNA levels were 281% higher in patients with early cholestasis (n=15) (p<0.01) and 321% higher in patients with biliary stricture (n=5) (p<0.05).

regulated in the early cholestasis group (281±40%, p=0.000) and the biliary group (231±61%, p<0.05) as compared to the control liver.

The MDR3 mRNA levels were higher in the early cholestasis group than those in the control group (738±116% vs 100%, p=0.000). In addition, the early cholestasis group showed a slightly stronger MDR3 mRNA expression than did the biliary stricture group (738% vs 423%, p>0.05). Finally, the mRNA levels of MDR1 in the biliary group and the early cholestasis group were 133±33% and 85±9% of those of the control donor liver, respectively (p>0.05).

DISCUSSION

For patients suffering within cholestatic disorders, the physiologic adaptive mechanisms include the induction of bile acid detoxification systems and the recruitment of alternative export pumps to deal withfor the cholestasis at the basolateral membrane, and thiswhich ultimately leads to increased urinary bile acid elimination.[6,10-12] In this study, it is interesting that there was significantly decreased expressions of the canalicular efflux transporters, including BSEP and MRP2, in the graft livers following LDLT ,while the expressions of basolateral uptake systems, such as NTCP, significantly increased in the early cholestasis group, as when compared with the matched donor control

livers. In contrast, alterations in the expressions of the transporter genes expression in the patients with primary biliary cirrhosis (PBC), which is a prototype of chronic cholestatic disease, were found to evolve in a diseases stage-dependent fashion.[13-15] Specifically, in the later PBC stages (III, IV), the NTCP expression was found to be down-regulated, while the MRP3 and MDR expressions were was up-regulated. Indeed, the expressions of BSEP and MRP2 were even found to increase during PBC stage III. In addition, the BSEP expression appeared to be relatively well preserved in the patients with extrahepatic biliary obstruction, and the MPR3 and MDR3 expressions were found to be up-regulated.[16] Taken together, these results suggest that the adaptive mechanism of the bile acid transporters did not work in the patients who suffered with having severe intrahepatic cholestasis following LDLT, as when compared to the patients with other types of cholestasis such as PBC and extrahepatic biliary obstruction (EHBO). This dysregulation of the expressions of the bile acid transporter genes may induce apoptosis in the hepatocytes due to the buildup of toxic bile acids.[17]

While changes of the transporter systems are the primary factors involved in hereditary cases of cholestasis, most alterations in the acquired cases of cholestasis are secondary.[18-20] The decreased expression of the transporter systems may explain, at least in part, the impairment of transporter function that results in the development or maintenance of cholestasis. However, we don't fully understand the reasons for these differences in the bile acid transporter expression that we observed in the cases of early cholestasis after LDLT, as compared to the other cholestatic disorders. It is possible that the limited hepatic bile acid uptake during cholestasis is a protective mechanism that reduces the hepatocellular bile acid overload. This would result in the expression of the primary basolateral bile acid uptake system (NTCP) being reduced in individuals with human cholestatic liver diseases.[21-23] Conversely, the results of this study demonstrated that significant up-regulation of NTCP mRNA occurred in the cases of early cholestasis following LDLT. This event would induce a significant increase of the hepatocellular damage. This discrepancy in the effects of the NTCP expression suggests that early cholestasis following LDLT is associated with many factors, including liver regeneration, inflammation and the effect of drugs, as compared with the other cholestatic diseases such as PBC. However, future studies are necessary to confirm the roles that the biliary transporters (BSEP, MRP2, MDR1, MDR2, NTCP) play in patients suffering with early cholstasis following LDLT.

Bile acid export via the canalicular membrane is primarily mediated by BSEP and MRP2.[14,24] The expressions of these transporters must be tightly controlled to prevent bile acid accumulation within the hepatocytes by increasing both the BSEP and MRP2 expressions.[25] However, the expressions of BSEP and MRP2 were decreased in patients with early cholestasis following LDLT. This indicates that the down-regulation of the canalicular export pumps, including BSEP and MRP2, may also potentiate the hepatocellular toxicity by enabling the accumulation of bile acids.

In this study, the patients with early cholestasis following LDLT showed some differences, as compared with the biliary stricture group. Cholestasis developed in the patients with biliary stricture approximately 6 to 12 months after LDLT. There are many possible etiologic factors that may be responsible for the early development of cholestasis in these patients, including arterial occlusion, prolonged cold preservation, rejection, CMV infection and ABO incompatible transplantation.[26,27] Microscopic analysis of the liver biopsy samples revealed that the site of cholestasis was primarily around the acinar zone 3 for the cases of early cholestasis, but this was primarily around the portal area for the cases in which cholestasis developed as a result of a biliary stricture. These findings indicate that the cholestasis in the biliary stricture group primarily resulted from the obstruction of bile flow in the large bile duct, which is similar to the development of cholestasis in response to EHBO.

However, early cholestasis primarily develops from the bile canaliculi, the small bile ductules and the hepatocytes. The suppression of the apical bile acid transporters BSEP and MRP2 was slightly lower in the biliary stricture group than that in the early cholestasis group. In addition, the levels of the expressions of the MDR1 and MPR3 genes tended to be higher in the cases of early cholestasis, but the levels of the expressions of the MDR1 and MPR3 genes were lower in the cases of cholestasis that developed as a result of biliary stricture.

The causes of early cholestasis following LDLT are known to be multifactorial, and these include ischemia-reperfusion injury, initial graft dysfunction, infections, small graft sizes, drugs and acute cellular rejection.[1] Sepsis was ruled out as a cause of the cholestasis in the patients who were evaluated in this study. However, bacterial infection is known to occur in 43% to 70% of cholestasis patients, and this usually occurs following transplantation.[28,29] The results of a previous study indicated that bacteremia that occurred within the first 30 days of transplantation was significantly correlated with the development of severe intrahepatic cholestasis.[30] In addition, many drugs that are known to have cholestatic side effects are administered to patients following LDLT, and particularly immunosuppressants, antibiotics

and antifungal agents. In this study, infection or drug toxicity could ruled out based on the clinical course, the evaluation of the liver biopsy and the culture studies. However, we could not completely rule out infection or drug toxicity as the causes of early cholestasis.

This study had several limitations. For example, the number of cases we evaluated was too small to provide definitive evidence of our results. Although only a few patients will develop early cholestasis following LDLT without any definitive causes such as biliary complications, early cholestasis is clinically important. Future studies should be conducted to clarify the basic mechanism(s) of early cholestasis. Additionally, because a follow up study, including repeated liver biopsies, was not performed, it is impossible to know if the alteration expressions of the bile acid transporter genes, which occurred in the cases of early cholestasis, recovered following the resolution of the cholestasis.

In conclusion, the results of this study suggest that early intrahepatic cholestasis following LDLT is associated with the suppressed expression of the bile acid transporter genes BSEP and MRP2, as well as with the overexpression of the NTCP gene. Moreover, these findings suggest that hepatocytes are not protected by the adaptive regulation of the bile acid transporter genes in the patients who are stricken with early severe intrahepatic cholestasis following LDLT. Further molecular studies are necessary to identify the exact mechanisms that are responsible for altering the expression of bile acid transporter genes in the patients who suffer with early intrahepatic cholestasis.

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