

Splenectomy for IFN therapy in LDLT

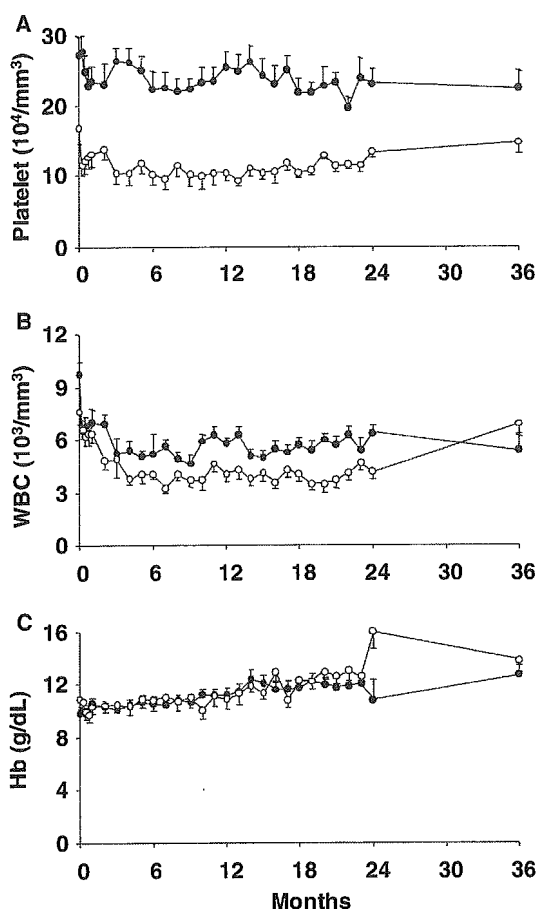


Fig. 1. Changes of platelet (A), white blood cell count (B) and hemoglobin (C). Levels after interferon therapy in group A (thick line with close circles) and group B (thin line with open circles). The bar represents a standard error value. There was a significant difference between the groups in the platelet count ($p = 0.008$).

Table 2. Timing [months after the start of interferon (IFN) therapy] and the reason of cessation of IFN therapy

Group	Patient	Timing	Reason
A	1	14	Renal dysfunction
	2	7	Depression
	3	7	Death caused by thrombotic thrombocytopenia
	4	18	Retransplantation because of cholestatic hepatitis
	5	19	Renal dysfunction
	6	3	Depression
B	1	4	Death caused by virus associated hemophagocytotic syndrome
	2	9	Thrombocytopenia
	3	6	Death because of hepatocellular carcinoma recurrence

hepatitis 18 months after the primary LDLT and died of liver failure 4 months after the retransplantation. Four patients in group A and three in group B completed the therapy. Eleven patients in

group A and eight in group B continued the therapy for 21 (range: 11–47) and 24 (range: 11–66) months, respectively.

Effect of genotype

In group A, HCV-RNA became negative in 44% (7/16) of HCV_{1b} patients and 60% (3/5) of the HCV_{non1b}. Median periods of treatment until the RNA level became negative was 15 (range: 1–18) months and 2 (range: 2–8) months in each group, respectively. There was no significant difference in the period by genotype ($p = 0.30$). In group B, HCV-RNA became negative in 17% (2/12) of HCV_{1b}, and 100% (2/2) of HCV_{non1b}.

Discussion

Preemptive IFN and ribavirin therapy to prevent cholestatic hepatitis has not been established. Only a few centers, including ours, report using this strategy (8, 10–13). Among the 39 patients who underwent preemptive IFN therapy after liver transplantation with or without splenectomy, we experienced cholestatic hepatitis in only one patient, which might indicate the possibility that long-term IFN and ribavirin therapy prevents the occurrence of cholestatic hepatitis. Gopal and Rosen (14) reported the results of IFN and ribavirin therapy in seven cholestatic hepatitis patients with only two patients who survived for an average of 32 months. They emphasized the importance of continuing the therapy indefinitely because the cessation of the therapy even after 12 months or more of treatment with sustained HCV-RNA negativity led to rapid recurrence of cholestatic hepatitis. IFN and ribavirin therapy might be worth continuing over the long term, especially in patients with HCV_{1b}. The preemptive therapy is effective in cases with lower HCV-RNA levels and less graft injury by the virus (11, 13). Accordingly, the treatment should be started within a short interval of transplantation.

The indications for simultaneous splenectomy in liver transplantation for reducing portal hypertension to protect the graft from congestion, especially in small left liver graft, or repairing portal flow regurgitation are established (15, 16). The effectiveness of splenectomy against thrombocytopenia is reported (9, 17). Several authors, however, have objected to perform splenectomy as a therapeutic option for thrombocytopenia because it might increase the risk of septic complications postoperatively, and instead recommend splenic artery ligation or radiologic partial splenic embolization (18–21). Several reports, however, suggest that

the indication of such ligation or embolization methods should also be considered with care because of the low success rate and risk of complications (22, 23). We previously reported the safety of concomitant splenectomy and several other centers report similar good results (9, 24). The results of the present study suggest that splenectomy is feasible for starting combination therapy early after transplantation and continuing for up to 4 yr with an acceptable morbidity rate.

The long-term effect of splenectomy as a therapeutic option for blood cytopenia because of portal hypertension remains unclear in patients undergoing IFN and ribavirin therapy. Randomized control trials to examine the risk and benefits of splenectomy for patients undergoing liver transplantation and combined therapy for hepatitis C are necessary.

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References

1. NEUMANN UP, BERG T, BAHRA M et al. Long-term outcome of liver transplants for chronic hepatitis C: a 10-year follow-up. *Transplantation* 2004; 77: 226.
2. RUSSO MW, GALANKO J, BEAVERS K, FRIED MW, SHRESTHA R. Patient and graft survival in hepatitis C recipients after adult living donor liver transplantation in United States. *Liver Transpl* 2004; 10: 340.
3. ROAYAIE S, SCHIANO TD, THUNG SN et al. Results of retransplantation for recurrent hepatitis C. *Hepatology* 2003; 38: 1428.
4. WATT KDS, LYDEN ER, McCASHLAND TM. Poor survival after liver retransplantation: is hepatitis C to blame? *Liver Transpl* 2003; 9: 1019.
5. MENON KVN, PTERUCHA JJ, EL-AMIN OM et al. Treatment of posttransplantation recurrence of hepatitis C with interferon and ribavirin: lessons on tolerability and efficacy. *Liver Transpl* 2002; 8: 623.
6. NEFF GW, O'BRIEN CB, CIROCCO R et al. Prediction of sustained virological response in liver transplant recipients with recurrent hepatitis C virus following combination pegylated interferon alfa-2b and ribavirin therapy using tissue hepatitis C virus reverse transcriptase polymerase chain reaction testing. *Liver Transpl* 2004; 10: 595.
7. ABDELMALEK MF, FIRPI RJ, SOLDEVILAP-PICO C et al. Sustained viral response to interferon and ribavirin in liver transplant recipients with recurrent hepatitis C. *Liver Transpl* 2004; 10: 199.
8. SUGAWARA Y, MAKUUCHI M, MATSUI Y et al. Preemptive therapy for hepatitis C virus after living donor liver transplantation. *Transplantation* 2004; 78: 1308.
9. CESCONE M, SUGAWARA Y, TAKAYAMA T et al. Role of splenectomy in living-donor liver transplantation for adults. *Hepatogastroenterol* 2002; 49: 721.
10. MAZZAFERRO V, REGALIA E, PULVIRENTI A et al. Prophylaxis against HCV recurrence after liver transplantation. Effect of interferon and ribavirin combination. *Transplant Proc* 1997; 29: 519.
11. MAZZAFERRO V, TAGGER A, SCHIAVO M et al. Prevention of recurrent hepatitis C after liver transplantation with early interferon and ribavirin treatment. *Transplant Proc* 2001; 33: 1355.
12. SINGH N, GAYOWSKI T, WANNSTEDT CF et al. Interferon-alpha for prophylaxis of recurrent viral hepatitis C liver transplant recipients: a prospective, randomized controlled trial. *Transplantation* 1998; 65: 82.
13. SHEINER PA, BROS P, KLION FM et al. The efficacy of prophylactic interferon alfa-2b in preventing recurrent hepatitis C after liver transplantation. *Hepatology* 1998; 28: 831.
14. GOPAL DV, ROSEN HR. Duration of antiviral therapy for cholestatic HCV recurrence may need to be indefinite. *Liver Transpl* 2003; 9: 348.
15. SHIMADA M, IJICHI H, YONEMURA Y et al. The impact of splenectomy or splenic artery ligation on the outcome of a living donor adult liver transplantation using a left lobe graft. *Hepatogastroenterology* 2004; 51: 625.
16. MASETTI M, SINISCALCHI A, PIETRI LD et al. Living donor liver transplantation with left liver graft. *Am J Transpl* 2004; 4: 1713.
17. ALTACA G, SCIGLIANO E, GUY SR et al. Persistent hypersplenism early after liver transplant: the role of splenectomy. *Transplantation* 1997; 64: 1481.
18. LÜSEBRINK R, BLUMHARDT G, LOHMANN R et al. Does concomitant splenectomy raise the mortality of liver transplant recipients? *Transpl Int* 1994; 7: S634.
19. TROISI R, COLLE I, VILERBERGHE HV, HESSE UJ, CUOMO O, HEPPTINNE B. Splenectomy and liver transplantation. *Transpl Proc* 2001; 33: 1500.
20. NEUMANN UP, LANGREHR JM, KAISERS U, LANG M, SCHMITZ V, NEUHAUS P. Simultaneous splenectomy increases risk for opportunistic pneumonia in patients after liver transplantation. *Transpl Int* 2002; 15: 226.
21. MATSUKURA A, KITA Y, HARIHARA Y et al. Is splenic artery ligation effective for thrombocytopenia early after liver transplantation? *Transpl Proc* 1999; 31: 2906.
22. LOHAR SC, ZAJKO AB, KONERU B, STEVENSON W, SUMKIN J. Splenic infarction complicating pediatric liver transplantation: incidence and CT appearance. *J Comput Assit Tomogr* 1990; 14: 362.
23. UFLACKER R, SELBY JB, CHAVIN K, ROGERS J, BALIGA P. Transcatheter splenic artery occlusion for treatment of splenic artery steal syndrome after orthotopic liver transplantation. *Cardiovasc Intervent Radiol* 2002; 25: 300.
24. KERCHER KW, CARBONELL AM, HENIFORD BT et al. Laparoscopic splenectomy reverses thrombocytopenia in patients with hepatitis C cirrhosis and portal hypertension. *J Gastrointest Surg* 2004; 8: 120.

Living Donor Liver Transplantation for Patients With Hepatitis C Virus Cirrhosis: Tokyo Experience

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Living donor liver transplantation is an alternative therapeutic option for patients with end-stage HCV cirrhosis because of the cadaveric organ shortage. Preliminary results, however, indicate that live donor grafts might be disadvantageous for HCV patients. Sixty-seven patients underwent living donor liver transplantation for HCV cirrhosis between 1996 and 2004. All the patients preemptively received antiviral therapy consisting of interferon alfa-2b and ribavirin, which was started approximately 1 month after the operation. The therapy continued for 12 months after the first negative HCV RNA test. The patients were then observed without the therapy for 6 months. The therapy was continued for at least 12 months, even when the HCV RNA test remained positive. The subjects were removed from the protocol if they could not continue the therapy for 12 months because of adverse effects or could not start the therapy because of early death. Twelve patients were removed from the protocol as a result of early death ($n = 9$) or cessation of the drug ($n = 3$). Another 16 patients are currently on the protocol. Of the remaining 39 patients, 16 patients (41%) had a sustained virologic response. The cumulative 5-year survival of the HCV-positive patients was 84%, which was comparable with that of patients negative for HCV ($n = 168$, 86%). The present preemptive antiviral protocol after living donor liver transplantation is safe and warrants a controlled study to confirm its benefit on graft survival.

Living donor liver transplantation (LDLT) is now a common alternative procedure to deceased donor liver transplantation (DDLT), which reduces waiting-time mortality in an era of deceased donor shortage. By June 2003, 1275 LDLT cases were recorded in the European Liver Transplantation Registry.¹ The 3-year graft survival rates were 71%, although the survival rates of HCV-positive patients are unknown. In the United States,² 1526 adult LDLT cases were performed by May 2004. HCV is the most common indication for LDLT, and the number of HCV-positive patients is stable, approximately 100 per year between 2000 and 2002. According to the Japanese Liver Transplantation Society,³ 1335 adult LDLT procedures were performed in Japan by

the end of 2003, and of these 297 (22%) were performed for HCV cirrhosis.

A current debate in the field of liver transplantation is the possibility of increased severity of recurrent HCV infection in LDLT patients. If HCV recurs earlier and more severely after LDLT, a specific strategy for preventing the detrimental effects of HCV on living donor grafts must be developed. Preemptive interferon therapy (prophylaxis) during the early post-transplantation period might reduce the incidence and severity of HCV recurrence. In the present study, we report our results of LDLT for chronic hepatitis C and discuss the feasibility of an antiviral protocol.

Patients and Methods

We performed preemptive therapy for LDLT patients with HCV infection. From 1996–2004, 67 patients underwent LDLT for HCV cirrhosis at the Tokyo University Hospital. The patients were 51 men and 16 women, and their ages ranged from 23–63 years (median, 55 years). The HCV genotype was 1b in 53 patients (79%). Forty-one patients (61%) had hepatocellular carcinoma. Our surgical technique for recipient and donor surgery is described elsewhere.⁴ All the patients received the same immunosuppressive regimens with tacrolimus (Prograf; Astellas Pharma Inc, Tokyo, Japan) and methylprednisolone as described previously.⁵

All the patients preemptively received antiviral therapy consisting of interferon alfa-2b and ribavirin, which was started approximately 1 month after the operation. The therapy was continued for 12 months after the first negative HCV RNA test. The standard regimen included interferon alfa-2b (3 million units [MU] \times 3 per week) and ribavirin (800 mg/day) for 6 months. The patients were then observed without the therapy for 6 months. The therapy was continued for at least 12 months, even if the HCV RNA test remained positive.

Therapy was discontinued when there was significant leukopenia ($<1500/\text{mL}$), thrombocytopenia ($<50,000/\text{mL}$) de-

Abbreviations used in this paper: DDLT, deceased donor liver transplantation; LDLT, living donor liver transplantation.

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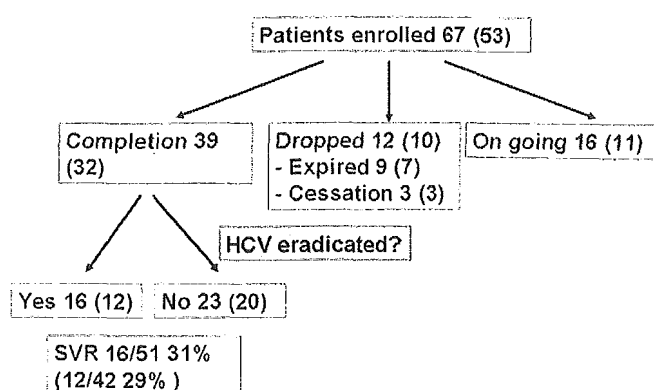


Figure 1. Results after preemptive antiviral therapy in University of Tokyo Hospital. Numbers in parentheses indicate those of genotype 1b. SVR, sustained viral response ratio. HCV eradicated? = Was the patient negative for HCV (<1000 copies/mL)?

spite application of granulocyte colony-stimulating factor (Gran; Sankyo Co Ltd, Tokyo, Japan), hemolytic anemia (hemoglobin <8 g/L), renal dysfunction (serum creatinine >2 mg/dL), depressive psychologic status, or general fatigue. The subjects were removed from the protocol if they could not continue the therapy for 12 months because of adverse effects or could not start the therapy as a result of early death.

Blood counts and liver function test results were checked every 2 weeks for the first month and at 4-week intervals thereafter. Serum samples were collected once a month for quantitative HCV RNA detection. Protocol liver biopsy was not performed. The log-rank test was used to compare the survival rate of the HCV-positive patients with the HCV-negative patients who underwent transplantation during the same period (n = 168).

Results

A total of 28 patients were excluded from the analysis (Figure 1). Twelve patients were removed from the protocol because of early death (n = 9) or because of drug cessation (n = 3). Another 16 patients are currently on the protocol and were therefore excluded from the analysis. Of the remaining 39 patients, 16 (41%) obtained a sustained virologic response. The cumulative 5-year survival of the HCV-positive patients was 84%, comparable with that of patients negative for HCV (n = 168, 86%).

Discussion

Because interferon is more effective in patients with a lower viral load,⁶ initiating preemptive therapy before peak viral loads are reached is a rational approach. There is, however, a theoretical risk of increasing cellular rejection, as observed in kidney and liver transplantation.⁷ Preemptive therapy during the early post-trans-

plantation period with interferon in combination with ribavirin has been attempted in DDLT.

In a case series by Mazzaferro et al,⁸ 36 recipients were treated with interferon alfa-2b (3 MU ×3 per week) and ribavirin (10 mg · kg⁻¹ · day⁻¹). Treatment was started a median of 18 days after the operation and continued for 11 months. After a median follow-up of 52 months, the 5-year patient survival was 88%. Serum HCV RNA clearance was obtained in 12 patients (33%). No further antiviral treatment was required because of negative HCV RNA in serum and normal liver histology for a median of an additional 36 months. In another study,⁹ 63 patients (<50% of screened cases) were randomized within 4 weeks after DDLT and treated for 48 weeks: 20 control subjects, 21 interferon alone, and 22 interferon and ribavirin. At 2 years, HCV RNA was negative in 13%, 13%, and 33%, respectively. Remarkably, there was no histologic recurrence in patients with a sustained viral response.

The association between LDLT and early HCV recurrence remains to be determined,¹⁰ although most of the recent reports suggest that living donor graft has no effect on short-term outcome or severity of virus recurrence. Reports from New York-Presbyterian Hospital¹¹ indicate that the time to diagnosis of recurrent HCV is significantly shorter in LDLT. Other data indicate that the 5-year survival of HCV patients (n = 69) who undergo LDLT is 64%, which is comparable with that of DDLT patients (n = 202, 69%). The multicenter adult to adult LDLT cohort study (A2ALL) might soon provide answers to questions about recurrent HCV after LDLT and DDLT.¹²

In areas where the cavaleric organ source is almost negligible, LDLT must be selected as a therapeutic option, regardless of any potential additional risk. The results of LDLT for HCV cirrhosis in our hospital were comparable with those for non-HCV patients. If living donor graft is associated with early HCV recurrence and consequently poorer graft survival, an aggressive antiviral protocol should be performed to improve the outcome of LDLT for HCV. The present data indicate that the protocol after LDLT is safe and warrants a controlled study to confirm its benefit for graft survival.

References

1. Data from European Liver Transplant Registry. Available at: http://www.eltr.org/publi/index_rv.php3. Accessed April 12, 2005.
2. Data from Organ procurement and transplantation network. Available at: <http://www.optn.org/latestData/advancedData.asp>. Accessed April 12, 2005.
3. The Japanese Liver Transplantation Society. Liver transplantation in Japan: registry by the Japanese Liver Transplantation Society (in Japanese). *Jpn J Transplant* 2004;39:634-642.

4. Sugawara Y, Makuuchi M, Sano K, et al. Vein reconstruction in modified right liver graft for living donor liver transplantation. *Ann Surg* 2003;237:180-185.
5. Sugawara Y, Makuuchi M, Kaneko J, et al. Correlation between optimal tacrolimus doses and the graft weight in living donor liver transplantation. *Clin Transplant* 2002;16:102-106.
6. Yamada G, Takatani M, Kishi F, et al. Efficacy of interferon alfa therapy in chronic hepatitis C patients depends primarily on hepatitis C virus RNA level. *Hepatology* 1995;22:1351-1354.
7. Samuel D. Hepatitis C, interferon, and risk of rejection after liver transplantation. *Liver Transpl* 2004;10:868-871.
8. Mazzaferro V, Tagger A, Schiavo M, et al. Prevention of recurrent hepatitis C after liver transplantation with early interferon and ribavirin treatment. *Transplant Proc* 2001;33:1355-1357.
9. Mazzaferro V, Schiavo M, Caccamo L, et al. Prospective randomized trial on early treatment of HCV infection after liver transplantation in HCV RNA positive patients (abstract). *Liver Transpl* 2003;9:C-36.
10. Sugawara Y, Makuuchi M. Should living donor liver transplantation be offered to patients with hepatitis C virus cirrhosis? *J Hepatol* 2005;42:472-475.
11. Gaglio PJ, Malireddy S, Levitt BS, et al. Increased risk of cholestatic hepatitis C in recipients of grafts from living versus cadaveric liver donors. *Liver Transpl* 2003;9:1028-1035.
12. Russo MW, Shrestha R. Is severe recurrent hepatitis C more common after adult living donor liver transplantation? *Hepatology* 2004;40:524-526.

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ORIGINAL ARTICLE

Is blood eosinophilia an effective predictor of acute rejection in living donor liver transplantation?*

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Keywords

acute cellular rejection, eosinophilia, liver transplantation, steroid.

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Introduction

In liver transplantation, acute cellular rejection (ACR) is still a major complication that can lead to mortality. Early diagnosis is necessary for prompt treatment, which must be based on liver biopsy. Several reports indicate a relationship between blood eosinophilia and acute rejection in liver transplantation [1–4]. Infiltration of eosinophils into the graft and peripheral blood eosinophilia might relate to ACR. In most studies, eosinophilia preceded

Summary

The association of blood eosinophilia with acute cellular rejection (ACR) after living donor liver transplantation has not been examined yet. The subjects were the 167 recipients who underwent liver biopsy (314 times). The blood eosinophil counts in the preoperative period ($n = 167$), 3 days before ($n = 314$) and on the day of biopsy ($n = 314$) were compared among the groups stratified by severity of ACR. Among 314 biopsy specimens, the 140 biopsy specimens were diagnosed with ACR. In the 140 ACR episodes, eosinophil counts before and after therapy was compared between the episodes that responded to therapy ($n = 80$) and those not ($n = 60$). The sensitivity and specificity of preoperative eosinophilia (eosinophil counts $>130 \text{ mm}^3$) to predict ACR was 33% and 65%, respectively. The eosinophil counts $>400 \text{ mm}^3$ 3 days before and on the day of biopsy was associated with the severity of ACR ($P < 0.0001$). The sensitivity to predict ACR was 26% and 33%, and the specificity, 94% and 93%, respectively. There was no significant difference in changes of eosinophil counts between the steroid-responders versus the nonresponders. The present results suggested the limited role of eosinophilia as a predictor of ACR after living donor liver transplantation.

ACR by 2–4 days [1,5]. One report demonstrated a close relationship between pretransplantation peripheral blood eosinophilia and postoperative ACR [6]. All of these reports, however, were based on data from deceased donor liver transplantation. In living donor liver transplantation (LDLT), the relation between eosinophilia and ACR has not been examined.

It is controversial that whether there is a difference in the frequency of ACR rejection between LDLT and deceased donor liver transplantation [7,8]. Some authors

reported lower incidence of steroid resistant [9] or late onset ACR [10] after LDLT. This might be due in part to the length of graft cold ischemic time [7] or the HLA haplotype matching in living-related donor cases [9]. The difference in the frequency and severity of ACR between deceased donor liver transplantation and LDLT led us to examine whether blood eosinophilia can predict ACR after LDLT.

Patients and methods

Patients

Subjects were 305 consecutive patients that underwent LDLT at our hospital. Two patients complicated by chronic rejection and eight patients who underwent emergent transplantation were excluded. Of the remaining 299 patients, biopsies were performed in 167 patients consisting 131 adults [47 ± 1.0 (mean \pm SE) in age] and 36 children (6.3 ± 1.0 years old). The indications for LDLT included HCV related cirrhosis ($n = 39$), hepatitis B virus related cirrhosis ($n = 14$), cirrhosis of other etiologies ($n = 7$), biliary atresia ($n = 37$), primary biliary cirrhosis ($n = 33$), primary sclerosing cholangitis ($n = 4$), autoimmune hepatitis ($n = 5$), fulminant hepatic failure ($n = 15$), metabolic diseases ($n = 7$) and others ($n = 6$).

Acute cellular rejection was diagnosed based on biopsy and graded into four classes according to the Banff scheme [11] [Grade 0 (G0): no evidence of rejection, Grade 1 (G1): mild rejection, Grade 2 (G2): moderate rejection, Grade 3 (G3): severe rejection; Fig. 1]. Postoperative immunosuppression was achieved with tacrolimus and methylprednisolone [12]. Tacrolimus was administered to control the trough level at approximately 16–18 ng/ml for the first week, and gradually tapered to 5–8 ng/ml over 6 months. Steroids were also tapered day by day from 3 mg/kg on the first postoperative day to 0.3 mg/kg on the fifteenth postoperative

day. The dose was then decreased slowly to 0.06 mg/kg over 6 months. When the diagnosis of ACR was confirmed, 20 mg/kg of methylprednisolone was administered, which was then tapered by reducing the dose by half each day until the same dose as before therapy was achieved.

Biopsy was performed when levels of all blood liver function tests, including transaminases, bilirubin, gamma-glutamyl transpeptidase and alkaline phosphatase, elevated. No protocol biopsy was performed.

Analysis

The relationship between preoperative eosinophilia and ACR stratified by grade was examined. Preoperative eosinophilia was defined as absolute eosinophil count (AEC) $>130 \text{ mm}^3$ [6]. The relationship of eosinophilia 3 days before or on the day of biopsy and ACR grouped by grade was examined. Here, the number of eosinophils was evaluated as AEC or relative eosinophil count (REC: $\text{AEC} \times 100/\text{total leukocyte count}$). Postoperative eosinophilia was defined as AEC more than $400/\text{mm}^3$ and/or REC more than 4% [3].

Pre- or post-treatment AEC, REC, and eosinophil count changes were compared between patients that responded to the treatment and those that did not. Treatment was judged successful when transaminase and bilirubin levels improved to normal levels and did not increase again during the following month. If liver dysfunction recurred again within 1 month, followed by biopsy-proven ACR, the treatment was defined as failed.

Statistics

Data were expressed as mean \pm SE. Sensitivity and specificity of eosinophilia was calculated for the prediction of ACR or improvement of ACR. AEC and REC were compared between groups using an unpaired *t*-test or one-way ANOVA. A *P*-value of <0.05 was considered statistically significant.

Results

Preoperative eosinophilia

An average of 2.2 biopsies were performed per patient. The interval between transplantation and biopsy was on 32 ± 2.0 days. The degree of ACR included G1 in 71, G2 in 18 and G3 in two patients. Other 76 patients showed only indeterminate evidence of ACR in every biopsy samples and were classified to G0. Preoperative AEC of the patients with and without postoperative ACR was $168 \pm 27/\text{mm}^3$ and $114 \pm 16/\text{mm}^3$, respectively ($P = 0.78$). There was no significant difference in REC (G0,

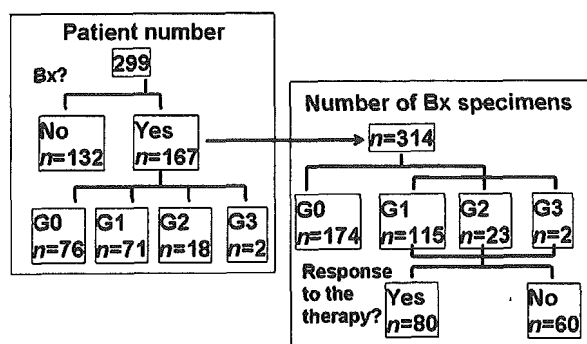


Figure 1 The numbers of the patients and liver specimens studied. Bx, liver biopsy.

2.6 ± 0.34%; G1, 2.9 ± 0.52%; G2, 3.7 ± 0.98%; $P = 0.54$) or AEC (G0, 114 ± 18/mm³; G1, 159 ± 27/mm³; G2, 217 ± 51/mm³; $P = 0.10$) among the G0–G2 grades of ACR (Fig. 2a). Two G3 specimens were excluded from the analysis. Preoperative eosinophilia predicted ACR with a sensitivity of 33% and a specificity of 65%, respectively (Table 1).

Eosinophilia 3 days before the biopsy

Eosinophil counts 3 days before the biopsy were available for 314 biopsy samples (Fig. 1), graded as G1 ($n = 115$) and G2 ($n = 25$). The other 174 samples showed indeterminate evidence of ACR and were classified to G0. The major findings the samples included nonspecific hepatitis with or without cholestasis ($n = 122$), congestion ($n = 15$), recurrent hepatitis C ($n = 15$) only mild lymphocyte infiltration or endothelialitis ($n = 5$), cholangitis ($n = 3$) and no abnormal findings ($n = 14$). REC and AEC 3 days before biopsy in patients complicated with ACR were 2.5 ± 0.3% and 234 ± 33/mm³, respectively. REC and AEC in patients without ACR were 0.8 ± 0.1% and 77 ± 12/mm³, respectively. When the biopsy samples were grouped according to the severity of ACR, there was a significant difference between the groups both in REC ($P < 0.0001$) and AEC ($P < 0.0001$; Fig. 2b). Eosinophilia (REC > 4%) 3 days before the biopsy predicted ACR with a sensitivity of 26% and a specificity of 94%, respectively (Table 1).

Eosinophilia on the day of biopsy

Eosinophil counts on the day of the biopsy were available for 314 biopsy samples. The REC and AEC on the day of the biopsy with findings of ACR were 3.3 ± 0.3% and 312 ± 35/mm³, respectively, being significantly higher than those without ACR ($n = 174$, 0.8 ± 0.1%, $P < 0.0001$ and 78 ± 13/mm³, $P < 0.0001$). When biopsy episodes were grouped according to the severity of ACR, there was a significant difference between groups both in REC ($P < 0.0001$) and AEC ($P < 0.0001$; Fig. 2c). Eosinophilia (REC > 4%) on the day of biopsy predicted ACR with a sensitivity of 33% and a specificity of 93%, respectively (Table 1).

Eosinophil count in response to treatment

Eosinophil count changes (count 1 week after treatment minus that just before treatment) could be calculated in the 140 biopsy episodes. Of these, 80 were responsive to steroid recycling therapy and 60 were resistant. Pretreatment REC and AEC were 2.8 ± 0.4% and 226 ± 35/mm³ in the responding group and 4.0 ± 0% and 426 ± 65/mm³ in the nonresponding group, respectively. Post-

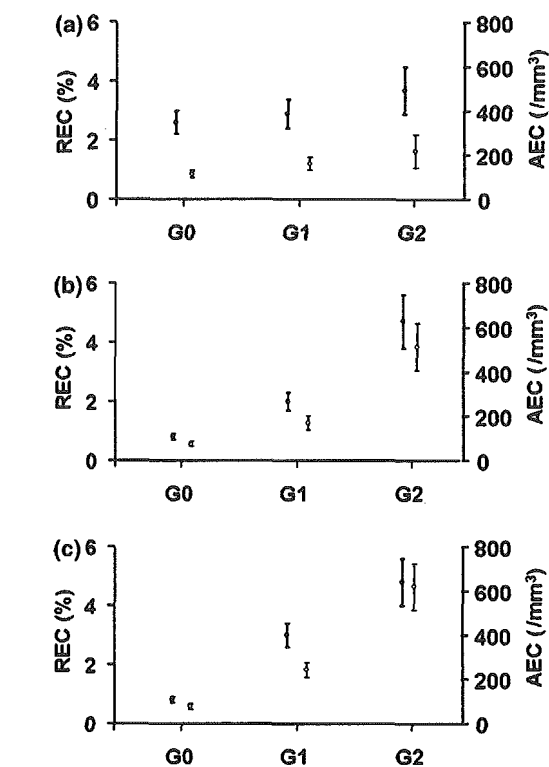


Figure 2 Relative (REC, thick bar and closed circle) and absolute eosinophil counts (AEC, thin bar and open circle) stratified by grade of rejection at preoperative (a) $n = 197$; 3 days before the biopsy (b) $n = 314$; and on the day of biopsy (c) $n = 314$. $P < 0.0001$ after comparison among the groups in the analyses of (b) and (c).

Table 1. Significance of eosinophil counts to predict acute cellular rejection.

Conditions	Events	Results	Sensitivity (%)	Specificity (%)
Pre-Tx	AEC > 130	ACR	33	65
Before Bx	REC > 4	ACR	26	94
	AEC > 400		20	95
On Bx	REC > 4	ACR	33	93
	AEC > 400		28	97
Before and after SRT	Decreased REC	Improvement of ACR	45	50
	Decreased AEC		50	43

Tx, transplantation; Bx, biopsy; SRT, steroid recycle therapy; ACR, acute cellular rejection; AEC, absolute eosinophil count; REC, relative eosinophil count.

treatment REC and AEC were 2.3 ± 0.5% and 176 ± 32/mm³ in the responding group and 2.6 ± 0.6% and 202 ± 55/mm³ in the nonresponding group, respectively. There was a significant difference between groups in the pretreatment AEC ($P = 0.04$), but not in pretreatment REC ($P = 0.07$), post-treatment REC ($P = 0.49$), or post-treatment AEC ($P = 0.48$).

Relative eosinophil count decreased in 36 and 30 treatments in the responding and nonresponding groups, respectively, whereas AEC decreased in 40 and 34 treatments. A decrease in REC or AEC predicted successful treatment of ACR with a sensitivity of 45% or 50% and a specificity of 50% or 43% (Table 1).

Discussion

Few studies have evaluated whether preoperative eosinophilia predicts ACR [6]. Nagral *et al.* [2] reviewed 129 biopsy cases. They demonstrated that there was no association between preoperative eosinophil count and the severity of ACR. They also demonstrated that AEC 1 or 2 days before or on the day of biopsy predicted ACR with low sensitivity (30.3–37.5%) and high specificity (83.3–91.8%). In our study also, eosinophilia both 3 days before and on the day of biopsy predicted ACR with low sensitivity and high specificity.

In contrast, Hughes *et al.* [13] emphasized that monitoring blood eosinophil count and serum eosinophil cationic protein was useful for early ACR diagnosis because they increase 2–3 days earlier than serum transaminase or alkaline phosphatase levels. Foster *et al.* [14] reported high sensitivity and specificity of blood eosinophilia in predicting ACR when they combined elevated serum transaminase or alkaline phosphatase levels. The exact reason for the discrepancy remains unclear, but might be due to a different dose of methylprednisolone for basal immunosuppression in our protocol: 3.0 mg/kg on the first postoperative day versus 1.5 mg/kg in Foster's report. The baseline eosinophil numbers might be decreased because of higher doses of steroid [15].

Our results indicated a higher pretreatment AEC in the steroid nonresponding ($426 \pm 65/\text{mm}^3$) compared with that of the responding group ($226 \pm 35/\text{mm}^3$, $P = 0.04$). They may support the phenomenon that the eosinophil count before or on the day of biopsy correlated well with the grade of ACR. A similar association was also reported by Barnes *et al.* [3] in liver transplantation and Trull *et al.* [15] in cardiac and lung transplantation. However REC was not a predictor of the response to the steroids, indicating the association between eosinophil counts before the treatment and the response to the treatment was not to be firm. Additionally the decrease in REC and AEC was not useful for predicting the effect of steroids on ACR in our series. Our results revealed a significant decrease in REC and AEC after steroid recycle therapy irrespective of the response to therapy. The finding might be explained by the hypothesis that steroids downregulate eosinophilia [16].

In summary, eosinophilia in the preoperative period, 3 days before and on the day of biopsy, predicted

consequent ACR with high specificity, but low sensitivity. The present results suggested the limited role of eosinophilia as a predictor of ACR after LDLT.

References

1. Foster PF, Sankary HN, Hart M, Ashman M, Williams JW. Blood and graft eosinophilia as predictors of rejection in human liver transplantation. *Transplantation* 1989; **42**: 72.
2. Nagral A, Quaglia A, Sabin CA, *et al.* Blood and graft eosinophils in acute cellular rejection of liver allografts. *Transpl Proc* 2001; **33**: 2588.
3. Barnes EJ, Abdel-Rehim MM, Goulis Y, *et al.* Applications and limitations of blood eosinophilia for the diagnosis of acute cellular rejection in liver transplantation. *Am J Transpl* 2003; **3**: 432.
4. Ben-Ari Z, Dhillon AP, Garwood L, Rolles K, Davidson B, Burroughs AK. Prognostic value of eosinophils for therapeutic response in severe acute hepatic allograft rejection. *Transplant Proc* 1996; **28**: 3624.
5. Manzarbeitia C, Rustgi VK, Jonsson J, Oyloek VK. Absolute peripheral eosinophilia. An early marker for resection in clinical liver transplantation. *Transplantation* 1995; **59**: 1358.
6. Dollinger MM, Plevris JN, Bouchier IAD, Harrison DJ, Hayes PC. Peripheral eosinophil count both before and after liver transplantation predicts acute cellular rejection. *Liver Transpl Surg* 1997; **3**: 112.
7. Jassem W, Koo DDH, Cerundolo L, Rela M, Heaton ND, Fuggle SV. Cadaveric versus living-donor livers: Differences in inflammatory markers after transplantation. *Transplantation* 2003; **76**: 1599.
8. Raymond R, Pierre W, Didier M, *et al.* Early hepatocyte, endothelial, and bile duct cell injury after pediatric liver transplantation from cadaveric or living-related donors. *Transplantation* 1998; **65**: 681.
9. Alonso EM, Piper JB, Echols G, Thistlethwaite JR, Whittington PF. Allograft rejection in pediatric recipients of living related liver transplants. *Hepatology* 1996; **23**: 40.
10. Toyoki Y, Renz JF, Mudge C, Ascher NL, Roberts JP, Rosenthal P. Allograft rejection in pediatric liver transplantation: comparison between cadaveric and living related donors. *Pediatr Transplant* 2002; **6**: 301.
11. Demetris AJ, Batts KP, Dhillon P, *et al.* Banff Schema for grading liver allograft rejection: An international consensus document. *Hepatology* 1997; **25**: 658.
12. Sugawara Y, Makuuchi M, Kaneko J, Ohkubo T, Imamura H, Kawarasaki H. Correlation between optimal tacrolimus doses and the graft weight in living donor liver transplantation. *Clin Transplant* 2002; **16**: 102.
13. Hughes VF, Trull AK, Joshi O, Alexander GJM. Monitoring eosinophil activation and liver function after liver transplantation. *Transplantation* 1998; **65**: 1334.

14. Foster PF, Sankary H, Williams JW. Study of eosinophilia and hepatic dysfunction as a predictor of rejection in human liver transplantation. *Transpl Proc* 1988; **20**: 676.
15. Trull A, Steel L, Cornelissen J, *et al.* Association between blood eosinophil counts and acute cardiac and pulmonary allograft rejection. *J Heart Lung Transplant* 1998; **17**: 517.
16. Meagher LC, Cousin JM, Seck JR, Haslett C. Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes. *J Immunol* 1996; **156**: 4422.

- [21] Azoulay D, Linhares MM, Huguet E, Delvart V, Castaing D, Adam R, et al. Decision for retransplantation of the liver: an experience-and cost-based analysis. *Ann Surg* 2002;6:713-721.
- [22] Rosen HR, Madden JP, Martin P. A model to predict survival following hepatic retransplantation. *Hepatology* 1999;29:365-370.
- [23] Rosen Hr, Prieto M, Casanovas-Taltavull T, et al. Validation and refinement of survival models for liver retransplantation. *Hepatology* 2003;38:460-469.
- [24] Yao FY, Saab S, Bass NM, Hirose R, Ly D, Terrault N, et al. Prediction of survival after liver retransplantation for late graft failure based on preoperative prognostic scores. *Hepatology* 2004;39:230-238.
- [25] Ghobrial RM. Retransplantation for recurrent hepatitis C in the model for end-stage liver disease era: how should we or shouldn't we? *Liver Transpl* 2003;9:1025-1027.

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Should living donor liver transplantation be offered to patients with hepatitis C virus cirrhosis?

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Cirrhosis secondary to hepatitis C virus (HCV) infection is a leading indication for living donor liver transplantation (LDLT) among adults in the United States, Europe, and Japan [1,2]. LDLT is now a common alternative procedure to deceased donor liver transplantation (DDLT), which reduces waiting-time mortality in an era of deceased donor shortage. By June 2003, 1275 LDLT cases were recorded in the European Liver Transplantation Registry [4]. The 3-year graft survival rates were 71%, although the survival rates of the HCV-positive patients are unknown. In the United States [5], 1526 adult LDLT cases were performed by May 2004. HCV is the most common indication for LDLT and the number of HCV-positive patients is stable, approximately 100 per year between 2000 and 2002. According to the Japanese Liver Transplantation Society [6], the number of LDLT patients has increased over time and 1063 adult LDLT procedures were performed in Japan by the end of 2002.

One of the hottest debates is the possibility of increased severity of recurrent HCV in LDLT patients. The benefit of LDLT might be offset if the outcome of LDLT for HCV patients is worse than that of DDLT. In the present manuscript, we review studies of LDLT and DDLT for HCV. Our results of LDLT for HCV are also reported herein.

1. Live donor as a risk factor for early recurrence?

1.1. Pros

At the American Transplant Congress held in Chicago in 2002, the UCLA group [7] reported that the time interval to

HCV recurrence in LDLT ($n=11$) was significantly shorter than in DDLT patients ($n=510$) (Table 1). Another group from the University of Colorado [8] reported that serum alanine aminotransferase and total bilirubin levels increased more rapidly after LDLT ($n=24$) than after DDLT ($n=41$). Those preliminary reports indicate that more intensive antiviral therapy might be necessary for recipients of living donor grafts. At the American Transplant Congress in 2004, two reports indicated a disadvantage of LDLT for HCV. One report from Barcelona [9,10] compared the results of LDLT ($n=22$) and DDLT ($n=95$). LDLT patients had younger donors, less graft steatosis, more frequent biliary complications, and earlier and more severe recurrent acute hepatitis. The other report from Cedars Sinai Medical Center [11] indicated that the time to diagnosis of recurrent HCV was significantly shorter in LDLT. Data from Colombia University [12,13] indicated that cholestatic hepatitis or severe recurrence occurs more frequently in LDLT.

A possible cause includes better HLA matching between donor and recipient. Because cellular immune reactions restricted by both HLA class I and II antigens are involved in the recognition of HCV peptides [14], HLA matching between donor and recipient could potentially increase damage to the graft from recurrent viral infections by facilitating host recognition of viral antigens [3]. Recently, a beneficial effect of a complete HLA-DQ mismatch was reported in 14 patients after transplantation for HCV cirrhosis [15]. Another possible cause might be related to liver regeneration [16]. In vitro, HCV internal ribosome entry site activity and replication were higher in actively dividing cells, and it is possible that viral translation is enhanced by factors that stimulate the regeneration of hepatocytes. Moreover, there are experimental data suggesting that liver regeneration induces LDL receptor

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Abbreviations: DDLT, deceased donor liver transplantation; HCV, hepatitis C virus; LDLT, living donor liver transplantation.

Table 1
Comparison between living and deceased donor liver transplantation (LDLT and DDLT) for hepatitis C virus cirrhosis

Study			N		Dif ^a	Protocol biopsy	Findings
Author	Year	Institution	LDLT	DDLT			
Ghobrial [7]	2002	UCLA	11	510	Yes	No	Rec in 86% of LDLT and 30% of DDLT during 1 year
Taniguchi [8]	2002	U. of Colorado	24	41	Yes	No	90 days (LDLT) and 168 days (DDLT) until Rec ($P < 0.05$) Higher AST level in LDLT until 30POD
Gaglio [12]	2003	Colombia U.	23	45	Yes	No	Cholestatic hepatitis in 17% of LDLT and 0% of DDLT ($P = 0.001$). No significant difference in incidence of Rec.
Garcia-Retorillo M [9,10]	2004	Hopital Clinic, Barcelona	22	95	Yes	Yes	Severe Rec in 41% of LDLT and 18% of DDLT. Higher ALT levels after 1 and 3 months
Pan [11]	2004	Cedars Sinai Medical C.	30	37	Yes	No	Earlier Rec in LDLT (161 days) than DDLT (295 days, $P < 0.006$). No significant difference in 1 year survival
Gaglio [13]	2004	Colombia U.	31	72	Yes	No	More frequent Rec in 87% of LDLT and 70% of DDLT ($P = 0.008$). No significant difference in time to Rec.
Trotter [17]	2001	Mount Sinai Medica C.	41	39	No	No	No significant difference in graft survival.
Schiffman [18]	2003	Virginia commonwealth U.	22	53	No	Yes	79% patient survival in LDLT and 91% in DDLT during 3 year (NS). No significant difference in inflammation score in liver specimen after 3 years
Vlierberghe [19]	2003	Ghent U.	17	26	No	No	No significant difference in frequency of (35% vs 38%), time (227 days vs 158 days) to Rec or 1 year survival.
Rodriguez-Luna [23,24]	2003, 2004	Mayo Clinic	9	20	No	Yes	No significant difference in frequency and severity of Rec after 4 and 12 months.
Vlierberghe [25]	2004	Ghent U.	17	26	No	No	Rec in 35% of LDLT and 38% of DDLT during 1 year ($P = 0.1$)
Gordon [20]	2004	Lahey Clinic Medical C.	19	47	No	No	No difference in severity of Rec and 3-year patient survival.
Fahmy [21]	2004	NYU Medical C.	33	52	No	No	No significant difference in incidence of Rec or cholestatic hepatitis.
Russo [22]	2004	UNOS data	279	3955	No	No	87% 1-year patient survival in both.
Bozorgzadeh [27]	2004	Rochester U.	35	65	No	No	Rec in 77% of LDLT and 72% of DDLT during 1 year (NS), 89% patient survival in LDLT and 75% in DDLT during 39 months (NS)

Abbreviations: Rec, Virus recurrence; AST, aspartate aminotransferase; ALT, alanine aminotransferase; C, Center; U, University; UNOS, United network for organ sharing; NS, not significant.

^a Difference in short-term outcomes or severity of virus recurrence between living and deceased donor liver transplantation.

expression, which might facilitate HCV entrance into the hepatocytes.

1.2. Cons

Some groups reported comparable data between LDLT and DDLT for HCV [17–21]. Russo and colleagues [22] compared patient and graft survival in recipients transplanted for chronic HCV who received a living donor organ ($n = 279$) and deceased donor organ ($n = 3955$) using the United Network for Organ Sharing liver transplant database. One-year patient survival was 87% in both groups and 2-year patient survival was 83 and 81% in the living donor group and deceased donor group ($P = 0.68$),

respectively. Analyses from the Mayo Clinic [23,24] and Gent University [25] failed to show a negative impact of live liver on the results of liver transplantation for HCV-related cirrhosis.

The data indicating poorer outcome with LRLT should be interpreted with caution because of the important clinical distinction between LDLT and DDLT recipients. At the time of transplantation, the LDLT group recipients are far less sick than their DDLT group counterparts [26]. The LDLT ($n = 35$) and DDLT ($n = 65$) data from a single institution, Rochester University, were examined [27]. Patient survival, graft survival, rate of HCV recurrence, severity of HCV recurrence, graft loss from HCV, and interval for recurrence in DDLT and LDLT were similar.

According to the data from Russo et al. [22], from 1999 to 2000 the 1-year patient survival in the LDLT group increased from 69 to 90% ($P=0.04$), and 1-year graft survival increased from 63 to 79% ($P=0.16$). In contrast, in the DDLT group, 1-year patient and graft survival did not substantially change from 1999 to 2000. As a result, 1-year survival rates became similar between the LDLT and DDLT groups in 2000. The results indicated an experience effect and learning curve on outcomes after LDLT for HCV. Therefore, initial reports indicating poorer results of LDLT might be due to technical problems from a lack of experience. Thus, recent data indicating similar results between LDLT and DDLT might be due to the increased experience with LDLT.

2. Management of HCV after LDLT

2.1. Therapy for recurrence in DDLT

If HCV recurs earlier and more severely after LDLT, a specific strategy for preventing the detrimental effects of HCV on living donor grafts must be developed. One strategy might be aggressive treatment for HCV. Treatment of recurrent HCV disease with interferon and ribavirin after DDLT is used in some centers [28–30] (see reviews from Berenguer and Negro in this FORUM). One standard regimen includes interferon-alpha 2b (3 MU \times 3 per week) and ribavirin (1000 mg/day) for 6 months. In a recent trial, polyethylene glycol-conjugated interferon therapy was used [31–33], with a sustained viral response rate ranging from 13 to 31%.

2.2. University of Tokyo experience [32]

We have performed preemptive therapy for LDLT patients for HCV. From July 1996 to July 2002, 23 patients underwent LDLT for HCV cirrhosis at the University of Tokyo Hospital. All the patients preemptively received antiviral therapy consisting of interferon-alpha 2b and ribavirin, which was started approximately 1 month after the operation. The therapy was given for 12 months after the first negative HCV-RNA test. The patients were then observed without the therapy for 6 months (Group 1). The therapy was continued for at least 12 months even when the HCV-RNA test remained positive (Group 2). The subjects were removed from the protocol if they could not continue the therapy for 12 months due to adverse effects or could not start the therapy due to early death.

Eight patients were removed from the protocol. Nine patients were assigned to Group 1 and the other six to Group 2. The sustained virologic response ratio was 39% (9/23). There was a significant difference between the groups in the histologic activity score 1 year after the therapy. The cumulative 3-year survival of the HCV-positive patients was 85%, comparable with that of patients

negative for HCV ($n=93$, 90%). The present study is preliminary, but the results warrant a randomized protocol to examine the feasibility of preemptive therapy for LDLT.

In conclusion the association between LDLT and early HCV recurrence remains to be determined, although most of the recent papers suggest that live donor graft had no effect on short-term outcome or severity of virus recurrence. The multicenter adult to adult LDLT cohort study (A2ALL) might soon provide some answers to the questions about recurrent HCV after LDLT and DDLT [33].

In areas where the cadaveric organ source is almost negligible, LDLT must be selected as a therapeutic option regardless of any potential additional risk. The results of LDLT for HCV cirrhosis in our hospital were comparable with those for non-HCV patients. If living donor graft is associated with early HCV recurrence and consequently poorer graft survival, an aggressive antiviral protocol could be performed to improve the outcome of LDLT for HCV.

In Western countries, the benefits of LDLT should not be overlooked and include reduced waiting-time mortality and a potential cure for some patients with hepatocellular carcinoma who might otherwise develop an incurable disease awaiting DDLT [41]. These real benefits of LDLT should be considered before making a decision about the risk of recurrent HCV with LDLT [33].

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References

- [1] Strader DB, Wright T, Thomas DL, Seeff LB. American association for the study of liver diseases. Diagnosis, management, and treatment of hepatitis C. *Hepatology* 2004;39:1147–1171.
- [2] Adam R, McMaster P, O'Grady JG, Castaing D, Klempnauer JL, Jamieson N, et al. Evolution of liver transplantation in Europe: report of the European liver transplant registry. *Liver Transpl* 2003;9:1231–1243.
- [3] Gane EJ, Portmann BC, Naoumov NV, Smith HM, Underhill JA, Donaldson PT, et al. Long-term outcome of hepatitis C infection after liver transplantation. *N Engl J Med* 1996;334:815–820.
- [4] Adam R, Lucidi V, Karam V. Liver transplantation in Europe: is there a room for improvement? 1st Forum in liver transplantation *J Hepatol* 2005.
- [5] Data from Organ procurement and transplantation network. <http://www.optn.org/latestData/advancedData.asp>.
- [6] The Japanese Liver Transplantation Society. Liver Transplantation in Japan. Registry by the Japanese Liver Transplantation Society. *Jpn J Transplant* 2004;38:401–408 (in Japanese).
- [7] Ghobrial RM, Amersi F, Farmer DG, Chen P, Anselmo DM, Baquerizo A, et al. Rapid and early HCV recurrence following adult living donor liver transplantation [Abstract]. *Am J Transplant* 2002; 2:63.

- [8] Taniguchi M, Wachs M, Back T, Trotter J, Kugelmas M, Everson G, et al. Hepatitis C recurrence in living donor liver transplantation [Abstract]. *Am J Transplant* 2002;2:138.
- [9] Garcia-Retortillo M, Forms X, Llovet JM, Navasa M, Massaguier A, Feliu A, et al. Hepatitis C virus (HCV) recurrence is an early and severe event in living donor liver transplantation (LDLT) [Abstract]. *Am J Transplant* 2004;4:294.
- [10] Garcia-Retortillo M, Forms X, Llovet JM, Navasa M, Feliu A, Massaguier A, et al. Hepatitis C recurrence is more severe after living donor compared to cadaveric liver transplantation. *Hepatology* 2004;40:699–707.
- [11] Pan SH, Nissen NN, Steven CD, Neda N, Tran TT, Poordad FF, et al. Comparison of rejection and hepatitis C recurrence in liver donor and deceased donor liver transplantation [Abstract]. *Am J Transplant* 2004;4:295.
- [12] Gaglio PJ, Malireddy S, Levitt BS, Lapointe-Rudow D, Lefkowitz J, Kinkhabwala M, et al. Increased risk of cholestatic hepatitis C in recipients of grafts from living versus cadaveric liver donors. *Liver Transpl* 2003;9:1028–1035.
- [13] Gaglio PJ, Jakob S, Novogrudsky A, Emond JC, Brown RS. Hepatitis C recurrence following liver transplantation: does histologic severity of inflammation in the explant correlate with recurrence? [Abstract]. *Am J Transplant* 2004;4:360.
- [14] Manez R, Mateo R, Tabasco J, Kusne S, Starzl TE, Duquesnoy RJ. The influence of HLA donor-recipient compatibility on the recurrence of HBV and HCV hepatitis after liver transplantation. *Transplantation* 1995;59:640–642.
- [15] Gretch D, Wile M, Gaur L. Donor-recipient match at the HLA-DQB locus is associated with recrudescence of chronic hepatitis following liver transplantation for end stage hepatitis C. *Hepatology* 1993;18:108A.
- [16] Olthoff KM. Hepatic regeneration in living donor liver transplantation. *Liver Transpl* 2003;9:S35–S41.
- [17] Trotter JF, Schiano T, Wach M, Kim-Schluger L, Bak T, Everson G, et al. Preliminary report: hepatitis C occurs earlier and is more severe in living donor liver transplant recipients [Abstract]. *Am J Transplant* 2001;1:316.
- [18] Schiffman ML, Stravitz RT, Contos MJ, Mills AS, Sterling RK, Luketic VA, et al. Histologic recurrence of chronic hepatitis C virus in patients after living donor and deceased donor liver transplantation. *Liver Transpl* 2004;10:1248–1255.
- [19] van Vlierberghe H, Troisi R, Ricciardi S, Colle I, Praet M, Conoscitore P, et al. Hepatitis C recurrence (HCV) does not worsen general outcome in adult living donor liver transplantation [Abstract]. *Am J Transplant* 2003;3:520.
- [20] Gordon FD, Pomfret EA, Pomposelli JJ, Keaveny A, Simpson MA, Lewis D, et al. The severity of recurrent hepatitis C (HCV) in living donor adult liver transplant (LDALT) recipient is the same as cadaver (CAD) recipients [Abstract]. *Am J Transplant* 2004;4:400.
- [21] Fahmy A, O'Mahony, Kaul H, Morgan GR, John D, Diffo T, et al. Living donor liver transplantation is safe and effective for hepatitis C recipients [Abstract]. *Am J Transplant* 2004;4:355.
- [22] Russo MW, Galanko J, Beavers K, Fried MW, Shrestha R. Patient and graft survival in hepatitis C recipients after adult living donor liver transplantation in the United States. *Liver Transpl* 2004;10:340–346.
- [23] Rodriguez-Luna H, Vargas HE, Peteris GD, Byrne T, Moss A, Mulligan D, et al. Hepatitis C virus recurrence in living donor liver transplantation vs. cadaveric liver transplantation [Abstract]. *Am J Transplant* 2003;3:160.
- [24] Rodriguez-Luna H, Vargas HE, Sharma P, Ortiz J, De Petris G, Balan V, et al. Hepatitis C virus recurrence in living donor liver transplant recipients. *Dig Dis Sci* 2004;49:38–41.
- [25] Vlierberghe H, Troisi R, Colle I, Ricciardi S, Praet M, de Hemptinne B. Hepatitis C infection-related liver disease: patterns of recurrence and outcome in cadaveric and living-donor liver transplantation in adults. *Transplantation* 2004;77:210–214.
- [26] Forman LM, Trotter JF, Emond J. Living donor liver transplantation and hepatitis C. *Liver Transpl* 2004;10:347–348.
- [27] Bozorgzadeh A, Jain A, Ryan C, Ornt D, Zand M, Mantry P, et al. Impact of hepatitis C viral infection in primary cadaveric liver allograft versus primary living-donor allograft in 100 consecutive liver transplant recipients receiving tacrolimus. *Transplantation* 2004;77:1066–1070.
- [28] Bizollon T, Ahmed SN, Radenne S, Chevallier M, Chevallier P, Parvaz P, et al. Long term histological improvement and clearance of intrahepatic hepatitis C virus RNA following sustained response to interferon-ribavirin combination therapy in liver transplanted patients with hepatitis C virus recurrence. *Gut* 2003;52:283–287.
- [29] Giostra E, Kullak-Ublick GA, Keller W, Fried R, Vanlemmens C, Kraehenbuhl S, et al. Ribavirin/interferon-alpha sequential treatment of recurrent hepatitis C after liver transplantation. *Transpl Int* 2004;17:169–176.
- [30] Abdelmalek MF, Firpi RJ, Soldevila-Pico C, Reed AI, Hemming AW, Liu C, et al. Sustained viral response to interferon and ribavirin in liver transplant recipients with recurrent hepatitis C. *Liver Transpl* 2004;10:199–207.
- [31] Sheiner PA, Boros P, Klion FM, Thung SN, Schluger LK, Lau JY, et al. The efficacy of prophylactic interferon alfa-2b in preventing recurrent hepatitis C after liver transplantation. *Hepatology* 1998;28:831–838.
- [32] Sugawara Y, Kaneko J, Akamatsu N, Kishi Y, Hata S, Kokudo N, et al. Living donor liver transplantation for end-stage hepatitis C. *Transpl Proc* 2004;36:1481–1482.
- [33] Russo MW, Shrestha R. Is severe recurrent hepatitis C more common after adult living donor liver transplantation? *Hepatology* 2004;40:524–526.

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Forum Feedback

MELD is not enough—enough of MELD?

To the Editor:

The recent review [1] on MELD confirms that interest in assessing prognosis in cirrhotic patients has been revived. However, several drawbacks of MELD, which deserve some comment, have not been considered.

Firstly, use of MELD for allocation is a 'justice' and not a 'utility' score, as it does not consider outcome after liver transplantation (LT). One reason is that donor factors are not considered [2]. As a result, both pre-LT MELD and change

in MELD [3] do not correlate with post-LT survival, with only a *c*-statistic of 0.58 in the UK [4]. In the USA, survival after transplantation was unchanged [1]. Secondly, the *c*-statistic for 3-month survival on the waiting list is as low as 0.75 [5]. Use of MELD outside the USA, has also given poor predictive accuracy in individual patients and poor generalisability [6].

Thirdly, the component variables of MELD may be difficult to assess, which may be one reason for poor

Clinical utility of quantitative RT-PCR targeted to α 1,4-*N*-acetylglucosaminyltransferase mRNA for detection of pancreatic cancer

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α 1,4-*N*-Acetylglucosaminyltransferase (α 4GnT) is a glycosyltransferase responsible for the biosynthesis of α 1,4-GlcNAc-capped *O*-glycans, and is frequently expressed in pancreatic cancer cells but not peripheral blood cells. In the present study, we tested the clinical utility of α 4GnT mRNA expressed in the mononuclear cell fraction of peripheral blood as a biomarker of pancreatic cancer. Total RNA isolated from the peripheral blood mononuclear cells from 55 pancreatic cancer patients, 10 chronic pancreatitis patients, and 70 cancer-free volunteers was analyzed quantitatively by reverse transcription-polymerase chain reaction with primers specific for α 4GnT, and the expression level of α 4GnT mRNA relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured. When the ratio of α 4GnT to GAPDH transcripts exceeded a defined cut-off value, patients were considered to have pancreatic cancer. By these standards, 76.4% of the pancreatic cancer patients were detected by this assay. A strong correlation was obtained between positivity in this assay and the expression of α 4GnT protein detected immunohistochemically in pancreatic cancer tissues resected subsequently, suggesting that α 4GnT mRNA detected in the peripheral blood is derived from circulating pancreatic cancer cells. Although increased levels of α 4GnT mRNA was detected in 40.0% of chronic pancreatitis patients and 17.1% of cancer-free volunteers, the expression levels were significantly lower than those seen in pancreatic cancer patients. These results suggest that quantitative analysis of α 4GnT mRNA expressed in the mononuclear cell fraction of peripheral blood will contribute to the detection of pancreatic cancer. (*Cancer Sci* 2006; 97: 119–126)

Pancreatic cancer is one of the most intractable malignancies.^(1,2) In particular, the 5-year survival rate of this neoplasm is the lowest of all types of cancer, and it is the fifth leading cause of cancer death in Japan.⁽³⁾ The poor prognosis of pancreatic cancer is largely attributable to the difficulty in diagnosis of the disease at relatively early stages as well as the highly invasive character of the cancer cells, regardless of the tumor size. In fact, the vast majority of pancreatic cancer patients are diagnosed at advanced stages associated with clinical manifestations such as jaundice and back pain, likely due to the limitation of tumor markers available for the diagnosis of pancreatic cancer at potentially

curable stages.⁽⁴⁾ Several well-established biomarkers, including CEA,⁽⁵⁾ CA19-9,⁽⁶⁾ DU-PAN-2^(7,8) and Span-1,⁽⁹⁾ are available for the detection of pancreatic cancer, but it is also true that these biomarkers are not elevated in certain numbers of pancreatic cancer patients. Thus, in order to detect pancreatic cancer more efficiently, it is necessary to identify novel biomarkers that will be useful for its diagnosis.^(10,11)

Mucous glycoproteins secreted from the gastroduodenal mucosa are heavily glycosylated and protect the mucosa against various pathogens and physical stresses. Among the oligosaccharides found in human gastrointestinal mucins, α 1,4-GlcNAc-capped *O*-glycan is unique because its expression in normal tissues is limited to gastric gland mucous cells, Brunner's gland of the duodenal mucosa and accessory gland of the pancreaticobiliary tract.⁽¹²⁾ Interestingly, this unique *O*-glycan is expressed frequently in neoplastic cells such as carcinomas of the stomach, bile duct and pancreas, as well as pancreatic intraepithelial neoplasia (PanIN-I, PanIN-II and PanIN-III),⁽¹³⁾ thus it is regarded as a tumor-associated carbohydrate antigen for these tumors.⁽¹²⁾ Recently we isolated a cDNA encoding human α 4GnT, which is responsible for the biosynthesis of α 1,4-GlcNAc-capped *O*-glycans, by expression cloning from a gastric mucosa cDNA library.⁽¹⁴⁾ We subsequently demonstrated that α 4GnT is expressed in the Golgi of gastric gland mucous cells and Brunner's glands in normal gastroduodenal mucosa as well as the Golgi of adenocarcinoma cells such as gastric, pancreatic and biliary tract cancers expressing α 1,4-GlcNAc-capped *O*-glycans.^(15–17)

Our previous study demonstrated that neither α 4GnT RNA nor protein is detectable in the normal peripheral blood cells.⁽¹⁷⁾ Thus, we quantitatively measured the expression levels of α 4GnT mRNA in the mononuclear cell fraction of peripheral blood obtained from gastric cancer patients using RT-PCR and demonstrated that this assay is useful to detect, as well as monitor, gastric cancer.⁽¹⁷⁾ The present study extends this assay for detection of pancreatic cancer using a technically

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Abbreviations: α 4GnT, α 1,4-*N*-Acetylglucosaminyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H. pylori, *Helicobacter pylori*; MTC, multiple tissue cDNA; ROC, receiver operating characteristic; RT-PCR, reverse transcription-polymerase chain reaction; TAMURA, 3'-6-carboxy-*N,N,N,N*-tetramethylrhodamine; TBS, Tris buffered saline.

improved modification. Specifically, levels of $\alpha 4GnT$ mRNA in the mononuclear cell fraction of peripheral blood from pancreatic cancer patients were determined quantitatively using multiplex PCR employed to detect simultaneously both $\alpha 4GnT$ and an internal standard gene in a single reaction tube.

Materials and Methods

Clinical samples

The present study involved 55 pancreatic cancer patients (34 men and 21 women; age range 45–92 years [mean \pm SE, 68.5 \pm 9.8 years]). For the reduction of jaundice, a drainage tube was placed in the common bile duct of 16 of 32 patients whose tumors were located in the pancreatic head, whereas none of the 23 patients whose tumor was located in the body or tail of the pancreas received such drainage. In addition to the pancreatic cancer patients, samples from 10 chronic pancreatitis patients (10 men; ages ranging from 55 to 75 years [65.8 \pm 7.6]) and 70 volunteers (70 men; ages ranging from 31 to 90 years [69.4 \pm 1.4]) were analyzed. These volunteers underwent a health screening and were verified to be cancer-free by routine examinations including abdominal ultrasonography. Written informed consent was obtained from all patients and volunteers prior to the study. Peripheral blood samples were taken from patients and volunteers. In pancreatic cancer patients, blood samples were collected before surgical resection of the primary tumor. When patients underwent endoscopic biopsy of the gastric mucosa, blood samples were taken minimally at 2-week intervals after biopsy.

In addition to the patients' samples, the Human Blood Fractions MTC Panel of the first-strand cDNA (Clontech, Palo Alto, CA, USA) was analyzed. This panel is composed of mononuclear cells (B cells, T cells and monocytes) pooled from 50 male or female Caucasians, resting CD8⁺ cells pooled from 33 male or female Caucasians, resting CD4⁺ cells pooled from 20 male or female Caucasians, resting CD14⁺ cells pooled from 36 male or female Caucasians, resting CD19⁺ cells pooled from 34 male or female Caucasians, CD19⁺ cells activated with pokeweed mitogen pooled from four male or female Caucasians, mononuclear cells activated with pokeweed mitogen and concanavalin A pooled from four male or female Caucasians, CD4⁺ cells activated with concanavalin A pooled from 12 male or female Caucasians, and CD8⁺ cells activated with phytohemagglutinin pooled from eight male or female Caucasians. These samples were analyzed using a real-time quantitative RT-PCR assay. In parallel, tissue specimens of pancreatic cancer obtained from 23 patients who subsequently underwent surgical operation for removal of primary tumors were examined by immunohistochemistry, and the tumor stage was classified according to the tumor node metastasis classification system.⁽¹⁸⁾ In addition, pancreatic tissue specimens of two cases operated for chronic pancreatitis were archived from the pathology files of Shinshu University Hospital, Matsumoto, Japan. The study protocol was approved by the Institutional Review Board of Shinshu University School of Medicine.

RNA extraction and cDNA synthesis

Five milliliters of peripheral blood was collected, treated with ethylene diamine tetraacetic acid to prevent coagulation,

and layered on 3 mL of Lymphprep (Nycomed Pharma, Oslo, Norway) in a 15-mL polypropylene tube. The tube was centrifuged at 2000g for 30 min at 20°C. The mononuclear cell fraction was transferred to a new tube, resuspended in 5 mL phosphate-buffered saline, and then centrifuged at 3000g for 5 min. Total RNA was isolated from the pellet using a RNeasy Mini kit (Qiagen, Valencia, CA, USA), followed by DNaseI treatment. After inactivation of DNaseI, 11 μ L of the DNaseI-treated RNA was incubated with 1 μ L of 10 mM dNTPs and 1 μ L of 0.5 mg/mL oligo(dT)₁₅ primer (Promega, Madison, WI, USA) at 65°C for 5 min. After chilling on ice, these mixed samples were then incubated with 4 μ L of 5 \times first strand buffer, 1 μ L of 0.1 M dithiothreitol, 1 μ L of RNase inhibitor (Promega), and 1 μ L of the reverse transcriptase SuperScript 2 (Invitrogen, Carlsbad, CA, USA) at 42°C for 1 h. The reaction was terminated by heating at 70°C for 15 min, and samples were then kept at –20°C until real-time quantitative RT-PCR analysis.

Real-time RT-PCR

Quantitation of $\alpha 4GnT$ mRNA expressed in peripheral blood mononuclear cells as well as the Human Blood Fractions MTC Panel was carried out using an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA) as described previously, with minor modifications.⁽¹⁷⁾ On the basis of the published human $\alpha 4GnT$ sequence,⁽¹⁴⁾ specific primer pairs and probes were designed using the Primer Express program (PE Applied Biosystems). Forward and reverse primers for human $\alpha 4GnT$ were 5'-GTTTTCTCTTCCC-TTTGGATATGA-3' (nucleotides +340 to +364; the first nucleotide of the initiation methionine codon is +1) and 5'-AGCTGATGTGGAGCCAGTTTCT-3' (nucleotides +427 to +448), respectively. These primers were designed to hybridize to different exons of the $\alpha 4GnT$ gene to avoid amplifying genomic DNA. The TaqMan probe was synthesized as 5'-TGGTACAATCAAATCAACGCCAGCGC-3' (nucleotides +397 to +422) by PE Applied Biosystems, and it carried a 5'-6-carboxyfluorescein reporter label and a TAMURA quencher group. To normalize $\alpha 4GnT$ mRNA expression levels, a housekeeping gene, *GAPDH*, was quantitatively analyzed simultaneously as a control. To construct a standard curve, 10-fold dilutions of the plasmid cDNA harboring $\alpha 4GnT$ (pcDNA1- $\alpha 4GnT$) ranging from 3 \times 10⁻² to 3 \times 10⁻¹⁰ μ g/mL, corresponding to 5 \times 10⁹ to 5 \times 10¹ copies/mL were prepared. Similarly, a 10-fold dilution of the plasmid cDNA harboring a partial cDNA sequence of *GAPDH* (pCR2.1-GAPDH), which was constructed as described previously,⁽¹⁷⁾ was prepared from 2.3 \times 10⁻² to 2.3 \times 10⁻¹⁰ μ g/mL, corresponding to 5 \times 10⁹ to 5 \times 10¹ copies/mL.

Multiplex PCR was carried out in 50 μ L of reaction mixture containing 3 μ L of cDNA sample, 25 μ L of 1 \times Universal PCR Master Mix (PE Applied Biosystems), 800 nM of the primer set for $\alpha 4GnT$, 80 nM of the primer for *GAPDH*, 125 nM of the TaqMan probe for $\alpha 4GnT$, and 100 nM of the TaqMan probe for *GAPDH* that carries the 5'-VIC reporter label and 3'-TAMURA quencher group (PE Applied Biosystems). Reaction tubes were placed in the ABI PRISM 7700 Sequence Analyzer, preheated at 95°C for 10 min and amplified for 50 cycles of 95°C for 15 s, followed by 60°C for 1 min. The abundance of $\alpha 4GnT$ mRNA and *GAPDH* mRNA was determined by comparison with the standard curves for

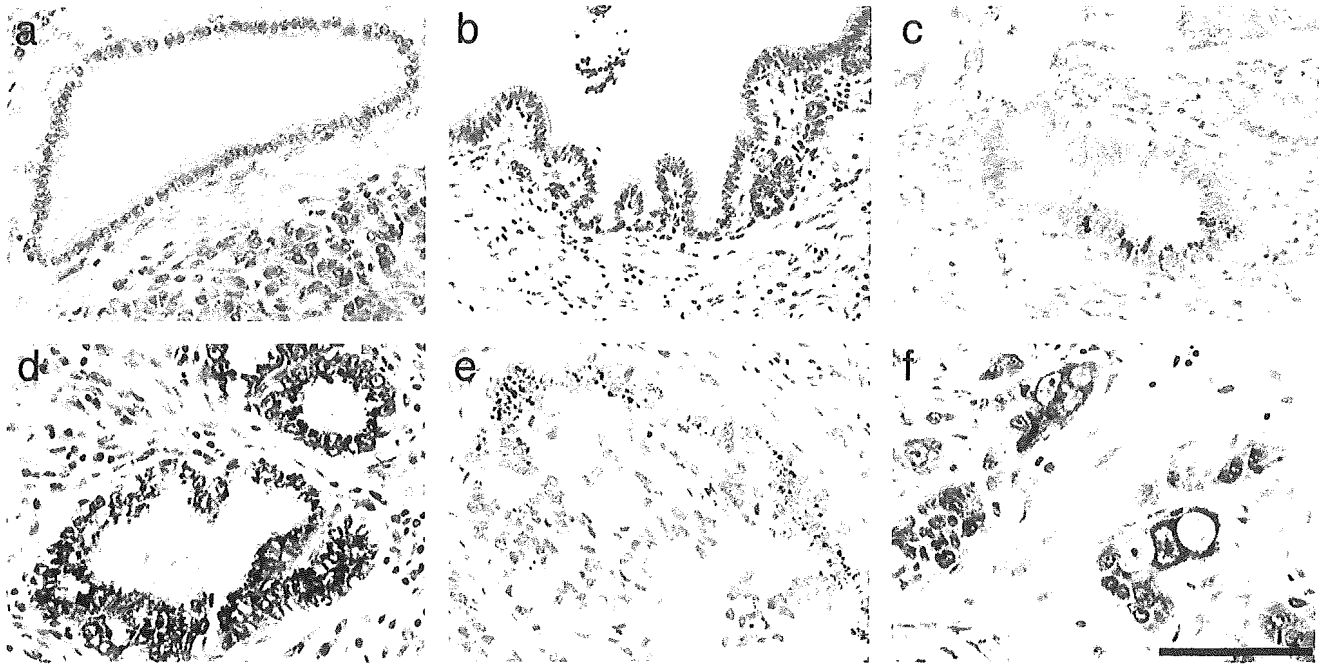


Fig. 1. Expression of α 4GnT protein in the normal and neoplastic pancreatic tissues. α 4GnT was detected by immunohistochemistry using the anti- α 4GnT antibody I17K. α 4GnT is not expressed in the normal pancreatic duct (a), whereas it is expressed in the Golgi region of pancreatic ducts exhibiting PanIN-IB (b). The α 4GnT protein is also expressed in the pancreatic ducts with PanIN-II found in chronic pancreatitis (c). In the pancreatic carcinoma, α 4GnT protein is detected in well differentiated (d), moderately differentiated (e), and poorly differentiated (f) adenocarcinomas. Scale bar = 100 μ M.

α 4GnT and GAPDH, respectively, and the relative expression level of α 4GnT mRNA was defined by multiplying the α 4GnT : GAPDH mRNA ratio by 1.0×10^7 . The assays were carried out in duplicate, and mean values of the two experiments were indicated.

Immunohistochemistry

To detect α 4GnT protein in pancreatic cancer cells, 23 cases of the resected pancreatic cancer tissues were subjected to immunohistochemistry with the monospecific anti- α 4GnT polyclonal antibody, I17K, as described previously.⁽¹⁶⁾ Briefly, 3 μ M-thick sections were deparaffinized and treated with 0.3% H_2O_2 in methanol and then blocked with 1% normal goat serum in TBS. The sections were incubated with the antibody for 1.5 h. After washing with TBS, sections were incubated with biotinylated antirabbit IgG and then horseradish peroxidase-labeled streptavidin. The peroxidase reaction was developed with a diaminobenzidine/ H_2O_2 solution, and counterstained with hematoxylin. In control experiments carried out by replacing the primary antibody with preimmune serum or omitting the primary antibody from the staining procedure, no specific staining was seen. Tissue specimens containing > 5% positively stained cancer cells were defined as positive, and the others were classified as negative according to previously described criteria.⁽¹⁹⁾

Enzyme immunoassay of biomarkers in patients' serum

Various biomarkers, including CEA, CA19-9, DU-PAN-2 and Span-1, in pancreatic cancer patients' serum were evaluated by enzyme immunoassay before surgery. CEA (cut-off value, 2.5 ng/mL) was measured using a CEA•Dainapack kit (Dainabot,

Tokyo, Japan), and CA19-9 (cut-off value, 37 U/mL) was measured using an AxSYM CA19-9•Dainapack kit (Dainabot). DU-PAN-2 (cutoff value, 150 U/mL) and Span-1 (cutoff value, 30 U/mL) were measured by SRL at Tokyo, Japan.

Statistics

Statistical analyses comparing two independent groups categorized by the clinicopathological variables of pancreatic cancer were carried out using the Mann-Whitney *U*-test. Similarly, comparisons among more than three groups were carried out using the Kruskal-Wallis test. These analyses were performed using StatView 5.0 software (Abacus Concepts, Berkeley, CA, USA). In addition, a cut-off value was determined by constructing a ROC curve using StatMate III (ATMS, Tokyo, Japan). Statistical association between the expression of α 4GnT protein in the resected pancreatic cancer tissues and the expression level of α 4GnT mRNA determined in the mononuclear cell fraction of peripheral blood was evaluated using Fisher's test (Abacus Concepts). In these analyses, *P*-values < 0.05 were considered to be statistically significant.

Results

Expression of α 4GnT protein in pancreatic cancer cells

In order to determine the expression of α 4GnT protein in pancreatic ductal adenocarcinoma cells, immunohistochemistry using the anti- α 4GnT antibody I17K was undertaken with normal and neoplastic tissues of the pancreas, which were resected surgically at the time of operation. In the normal pancreas, α 4GnT was not detected in the main or interlobular pancreatic ducts (Fig. 1a). By contrast, α 4GnT protein was

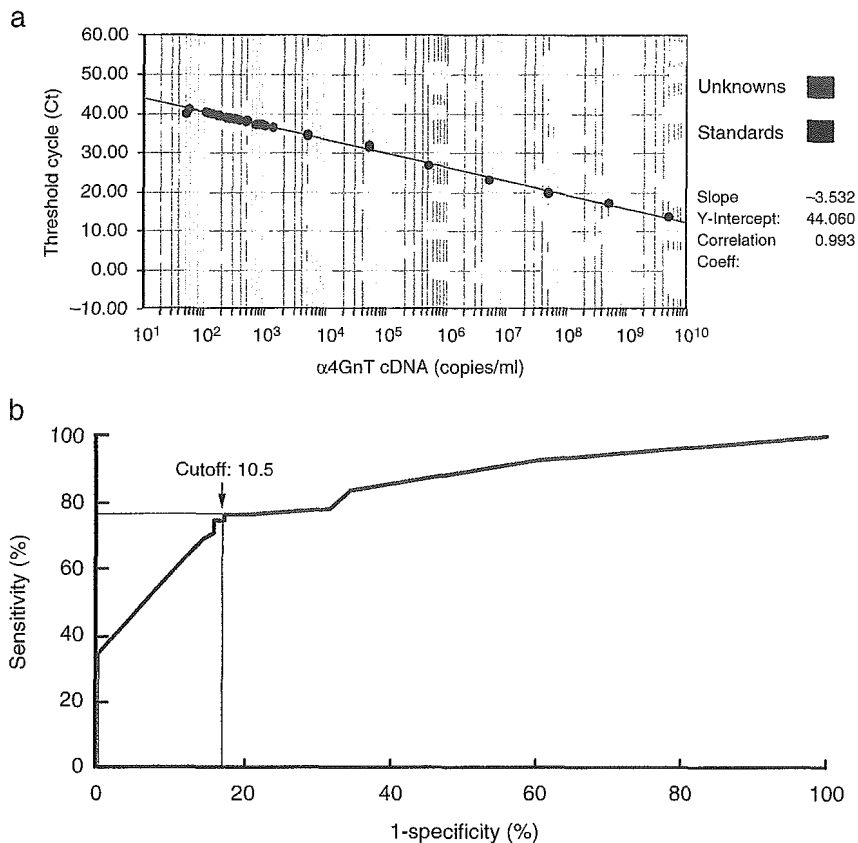


Fig. 2. Quantitative RT-PCR assay targeting $\alpha 4\text{GnT}$ mRNA. (a) A standard curve for $\alpha 4\text{GnT}$ was constructed by plotting serially diluted $\alpha 4\text{GnT}$ cDNA, pcDNA1- $\alpha 4\text{GnT}$ (black dots), where unknown samples from patients or cancer-free volunteers are indicated as red dots. (b) ROC curve was created by plotting the expression level of $\alpha 4\text{GnT}$ mRNA in the peripheral blood from 55 pancreatic cancer patients and 70 cancer-free volunteers. Arrow denotes the cutoff value of 10.5, which best discriminates pancreatic cancer patients from cancer-free volunteers with 76.4% sensitivity and 82.9% specificity.

associated with the Golgi region of pancreatic ducts with PanIN-IB (Fig. 1b). $\alpha 4\text{GnT}$ protein was also expressed in the pancreatic ducts with PanIN-II found in the inflammatory lesions of chronic pancreatitis in both of the two cases examined (Fig. 1c). In pancreatic cancer, $\alpha 4\text{GnT}$ was detected in the Golgi of adenocarcinoma cells in 73.9% of 23 patients, irrespective of histological tumor type; that is, five of nine patients with well-differentiated adenocarcinoma (Fig. 1d), seven of eight patients with moderately differentiated adenocarcinoma (Fig. 1e) and five of six patients with poorly differentiated adenocarcinoma (Fig. 1f) were positive for $\alpha 4\text{GnT}$ protein in cancer tissues.

Construction of a standard curve for the quantitative RT-PCR assay

The standard curve for $\alpha 4\text{GnT}$ mRNA was constructed using 10-fold dilutions of $\alpha 4\text{GnT}$ cDNA, pcDNA1- $\alpha 4\text{GnT}$ (Fig. 2a). By defining the cycle number where fluorescence reached a detection threshold as Ct, we obtained a strong linear relationship between Ct and the log of the cDNA concentration. Based on the standard curve, levels of $\alpha 4\text{GnT}$ mRNA ranging from 5×10^1 to 5×10^9 copies/mL were detected in a reaction tube. Similarly, GAPDH mRNA was detected ranging from 5×10^1 to 5×10^9 copies/mL based on the standard curve for GAPDH constructed using 10-fold dilutions of pCR2.1-GAPDH. Using these standard curves, the expression level of $\alpha 4\text{GnT}$ mRNA relative to that of GAPDH mRNA was determined.

Determination of a cut-off value distinguishing pancreatic cancer patients from cancer-free volunteers

To most efficiently discriminate pancreatic cancer patients from cancer-free volunteers, a ROC curve was constructed (Fig. 2b). Thus, the $\alpha 4\text{GnT}$:GAPDH mRNA ratios multiplied by 1.0×10^7 were defined as the expression level of $\alpha 4\text{GnT}$, and the values determined in the mononuclear cell fraction of the peripheral blood from 55 patients with pancreatic cancer versus 70 cancer-free volunteers were plotted. By defining the cut-off value as 10.5, the optimal combination of 76.4% for sensitivity and 82.9% for specificity was obtained. Thus, we regarded a value as positive when expression levels of $\alpha 4\text{GnT}$ mRNA greater than 10.5 were obtained in this assay.

Determination of the expression levels of $\alpha 4\text{GnT}$ mRNA in peripheral blood samples from pancreatic cancer patients and cancer-free volunteers

Based on the criterion that the expression level of $\alpha 4\text{GnT}$ mRNA should exceed the cut-off value of 10.5 for a positive result in this assay, we determined the expression levels of $\alpha 4\text{GnT}$ mRNA in the mononuclear cell fraction of peripheral blood isolated from 55 pancreatic cancer patients and 70 cancer-free volunteers (Fig. 3).

In pancreatic cancer, 42 (76.4%) of 55 patients examined were positive for this assay, and the expression level of $\alpha 4\text{GnT}$ mRNA was 37.50 ± 5.44 (mean \pm SE). The expression level of $\alpha 4\text{GnT}$ transcripts was then evaluated by clinicopathological variables including tumor location, stage, venous invasion,

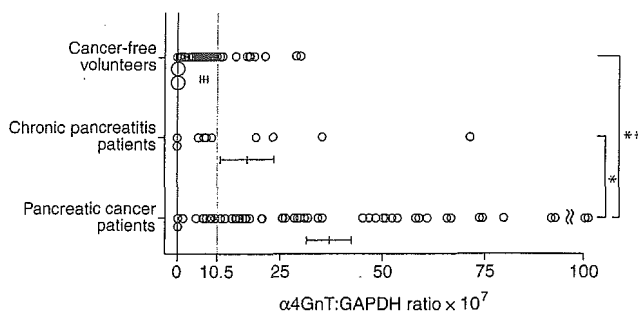


Fig. 3. Scatter plots indicating the expression level of $\alpha 4\text{GnT}$ mRNA in the mononuclear cell fraction of peripheral blood measured by the quantitative RT-PCR. Small and large circles represent one and 10 individuals, respectively. Horizontal bars indicate mean \pm SE. * $P < 0.05$, ** $P < 0.001$.

lymphatic invasion and lymph node metastasis determined at subsequent surgical operation. Although the frequency of the positive patients and the expression level of $\alpha 4\text{GnT}$ seemed to be associated with tumor progression, no significant statistical differences were seen in any clinicopathological variables examined (Table 1).

In addition, the expression level of $\alpha 4\text{GnT}$ mRNA in the mononuclear cell fraction of blood samples from cancer-free volunteers was determined. Of the 70 cancer-free volunteers examined, 12 (17.1%) volunteers were found to be positive for this assay, but the expression level of $\alpha 4\text{GnT}$ transcripts was 7.2 ± 0.9 , which was significantly lower than that seen in pancreatic cancer patients ($P < 0.001$).

We then tested whether activated lymphocytes express $\alpha 4\text{GnT}$ mRNA aberrantly by using the Human Blood Fractions MTC Panel, and it was shown that $\alpha 4\text{GnT}$ mRNA was not detectable in any of the blood fractions examined, including activated lymphocytes.

Detection of $\alpha 4\text{GnT}$ mRNA in peripheral blood samples from chronic pancreatitis patients

We next measured the expression level of $\alpha 4\text{GnT}$ in the mononuclear cell fraction of peripheral blood isolated from chronic pancreatitis patients (Fig. 3). Of the 10 patients examined, four (40.0%) were classed as positive by exceeding the defined cutoff of 10.5. However the expression level of $\alpha 4\text{GnT}$ mRNA was found to be 17.87 ± 6.98 , which was again significantly lower than that seen in pancreatic cancer patients ($P < 0.05$). Statistically significant differences between expression levels were not seen between cancer-free volunteers and chronic pancreatitis patients.

Comparison of $\alpha 4\text{GnT}$ mRNA with well-characterized biomarkers in pancreatic cancer patients

The results of the real-time PCR analysis of $\alpha 4\text{GnT}$ mRNA expressed in the mononuclear cell fraction of peripheral blood from the pancreatic cancer patients were then compared with the results of enzyme immunoassays for well-characterized biomarkers including CEA, CA19-9, DU-PAN-2 and Span-1. As shown in Table 2, more than 74% of pancreatic patients were positive for either $\alpha 4\text{GnT}$ or CA19-9, and CEA and DU-PAN-1 were found to less frequently detect pancreatic cancer compared with $\alpha 4\text{GnT}$ and CA19-9.

Table 1. Frequency of pancreatic cancer patients positive for the $\alpha 4\text{GnT}$ assay and correlation between expression levels of $\alpha 4\text{GnT}$ mRNA in peripheral blood mononuclear cells and clinicopathological variables

Variable	Frequency of positive patients [†]		$\alpha 4\text{GnT}$ mRNA [‡] (mean \pm SE)	P-value
	n	%		
Tumor location				
Head	25/32	78.1	37.95 ± 7.03	0.9728 [§]
Body and tail	17/23	73.9	36.87 ± 8.78	
Tumor stage				
0	0/1	0	5.290	0.4571 [¶]
II	2/3	66.7	29.44 ± 11.67	
III	7/8	87.5	32.85 ± 8.55	
IV	33/43	76.7	39.68 ± 6.72	
Venous invasion				
Negative	3/5	60.0	29.29 ± 11.20	0.6954 [§]
Positive	17/19	89.5	36.97 ± 10.36	
Lymphatic invasion				
Negative	1/3	33.3	11.81 ± 4.82	0.0606 [§]
Positive	19/21	90.5	38.74 ± 9.44	
Lymph node metastasis				
Negative	5/7	71.4	28.78 ± 9.93	0.8241 [§]
Positive	15/17	88.2	38.09 ± 11.32	

[†]Expression levels greater than 10.5 were defined as positive.

[‡] $\alpha 4\text{GnT}$:GAPDH mRNA ratios multiplied by 1.0×10^7 are indicated.

[§]Analyzed using the Mann-Whitney U-test. [¶]Analyzed using the Kruskal-Wallis test.

We then tested whether a combined assay with $\alpha 4\text{GnT}$ and another biomarker would detect pancreatic cancer patients more efficiently than the enzyme immunoassays targeting single biomarkers (Table 3). Notably, it was found that more than 86% of pancreatic cancer patients were detected when the $\alpha 4\text{GnT}$ assay was combined with enzyme immunoassay for CEA, CA19-9, DU-PAN-2 or Span-1. In particular, 96.4% of pancreatic cancer patients were positive for either $\alpha 4\text{GnT}$ mRNA or Span-1 or both, whereas 71.4% of the patients were positive for Span-1 alone.

Detection of $\alpha 4\text{GnT}$ protein in resected pancreatic cancer tissues

Transcripts of $\alpha 4\text{GnT}$ are not detectable in peripheral blood cells, including leukocytes, lymphocytes and monocytes.⁽¹⁷⁾ Thus, it is possible that $\alpha 4\text{GnT}$ mRNA detected in the mononuclear cell fraction of peripheral blood from pancreatic cancer patients is derived from circulating pancreatic cancer cells expressing $\alpha 4\text{GnT}$ mRNA. To test this hypothesis, the results of real-time RT-PCR of $\alpha 4\text{GnT}$ mRNA expressed in the peripheral blood were compared with those of $\alpha 4\text{GnT}$ protein expressed in 23 cases of the subsequently resected pancreatic cancer tissues by immunohistochemistry with the anti $\alpha 4\text{GnT}$ antibody I17K. In 19 patients positive for $\alpha 4\text{GnT}$ transcripts in the peripheral blood, 17 were also positive for $\alpha 4\text{GnT}$ protein in the resected pancreatic cancer tissues. By contrast, $\alpha 4\text{GnT}$ protein was not detected in pancreatic cancer tissues of three of four patients who were also negative for the $\alpha 4\text{GnT}$ mRNA assay. These results indicate a significant association between $\alpha 4\text{GnT}$ mRNA in the peripheral blood

Table 2. Frequency of pancreatic cancer patients detected using assays for α 4GnT mRNA, CEA, CA19-9, DU-PAN-2 and SPan-1

Tumor stage	α 4GnT mRNA (> 10.5)		CEA (> 2.5 ng/mL)		CA19-9 (> 37 U/mL)		DU-PAN-2 (> 150 U/mL)		SPan-1 (> 30 U/mL)	
	n	%	n	%	n	%	n	%	n	%
0	0/1	0	0/1	0	0/1	0	NE	NE	NE	NE
II	2/3	66.7	0/3	0	0/3	0	0/2	0	0/2	0
III	7/8	87.5	3/8	37.5	6/8	75.0	1/8	12.5	2/7	28.6
IV	33/43	76.7	23/41	56.1	34/42	82.9	16/22	72.7	18/19	94.7
Total	42/55	76.4	26/53	49.1	40/54	74.1	17/32	53.1	20/28	71.4

NE, not evaluated.

Table 3. Frequency of pancreatic cancer patients detected using combined assays[†]

Biomarker	CEA (> 2.5 ng/mL)		CA19-9 (> 37 U/mL)		DU-PAN-2 (> 150 U/mL)		SPan-1 (> 30 U/mL)	
	n	%	n	%	n	%	n	%
α 4GnT mRNA	46/53	86.8	48/54	88.9	30/32	93.8	27/28	96.4
CEA	-	-	43/53	81.1	24/32	75.0	21/28	75.0
CA19-9	-	-	-	-	27/32	84.4	23/28	82.1
DU-PAN-2	-	-	-	-	-	-	21/28	75.0

[†]Frequency of the patients positive for either or both biomarkers combined is indicated.

and α 4GnT protein in pancreatic cancer tissues ($P = 0.0209$), suggesting that α 4GnT mRNA detected in patients' peripheral blood is derived from circulating pancreatic cancer cells.

Discussion

α 1,4-*N*-Acetylglucosaminyltransferase is a glycosyltransferase that mediates the transfer of GlcNAc with an α 1,4-linkage from UDP-GlcNAc to β Gal residues, forming α 1,4-GlcNAc-capped *O*-glycans.⁽¹⁴⁾ As shown in our previous studies and confirmed here, α 4GnT is expressed frequently in pancreatic cancer cells as well as in gastric cancer cells, but not in peripheral blood cells.^(15,17) Therefore, we used quantitative RT-PCR to determine the expression level of α 4GnT mRNA in tumor cells circulating in the peripheral blood of pancreatic cancer patients. We primarily defined the cut-off value as 10.5 for this assay, based on the ROC curve, and could detect 76.4% of 55 pancreatic cancer patients. The significant correlation between the expression level of α 4GnT mRNA in the peripheral blood detected by the RT-PCR assay and α 4GnT protein detected in resected pancreatic cancer tissues by immunohistochemistry strongly suggests that α 4GnT mRNA detected in the peripheral blood is derived from circulating pancreatic cancer cells. Although 40% of 10 chronic pancreatitis patients and 17.1% of 70 cancer-free volunteers were also positive by this assay, the expression levels of α 4GnT mRNA in both groups were significantly lower than those seen in pancreatic cancer patients. These results indicate the clinical utility of real-time RT-PCR targeted to α 4GnT mRNA for detection of pancreatic cancer.

The present study also revealed that the location of the pancreatic tumor does not alter the results of the assay (Table 1). It is known that early detection of pancreatic cancer occurring in the tail and body of the pancreas can be particularly difficult because jaundice, which is frequently associated with pancreatic

head cancer, is not evident unless the common bile duct is affected by the tumor.⁽¹¹⁾ Thus, the assay demonstrated here will likely contribute to early detection of pancreatic body and tail cancers that are not associated with jaundice.

In the present study, we have also shown that the expression level of α 4GnT mRNA in the peripheral blood from pancreatic cancer patients is elevated in a manner correlated with tumor stage (Table 1), suggesting that the number of cancer cells entering the peripheral blood is increased as the tumor progresses. Most recently, we have shown that α 1,4-GlcNAc-capped *O*-glycans secreted from gastric gland mucous cells function as an antibiotic against *H. pylori* infection.⁽²⁰⁾ The role of these unique *O*-glycans expressed on pancreatic cancer cells remains unknown, and thus further study will be required to address this problem.

There are several biomarkers for pancreatic cancer, including CEA,⁽⁵⁾ CA19-9,⁽⁶⁾ DU-PAN-2^(7,8) and Span-1.⁽⁹⁾ Among them, CA19-9 is the most widely used in screening and monitoring of the disease.⁽²¹⁻²⁴⁾ We compared α 4GnT with other biomarkers (including CA19-9) and found that the frequency of pancreatic cancer patients detected by α 4GnT was much the same as that detected by CA19-9 (Table 2). The same analysis also revealed that DU-PAN-2 and CEA detected pancreatic cancer patients less frequently than α 4GnT, CA19-9 and Span-1. It is noteworthy that two of three patients at stage II were positive for α 4GnT mRNA, suggesting the possible usefulness of α 4GnT mRNA for the early detection of pancreatic cancer. Further study on a larger number of patients with stages 0, I and II will be required to prove this possibility.

The present study demonstrated that the frequency of pancreatic cancer patients detected using enzyme immunoassays for CEA, CA19-9, DU-PAN-2 and Span-1 was increased substantially when combined with the α 4GnT assay (Table 3). It is generally accepted that the quantitative RT-PCR assay requires much time and cost compared with enzyme immunoassay.