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***Original Article***

# Incidence of and risk factors for bile duct stones after living donor liver transplantation: An analysis of 100 patients

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*Aim:* Although bile duct stone (BDS) is one of the biliary complications of liver transplantation, analytical studies, particularly on living donor liver transplantation (LDLT) cases, are rare. This study aimed to clarify the incidence of and risk factors for BDS following LDLT.

*Methods:* We retrospectively reviewed the medical records of 100 patients who underwent LDLT at our institute from August 2000 to May 2012, and analyzed their clinical characteristics and risk factors for BDS.

*Results:* Of these, 10 patients (10.0%) developed BDS during the observation period. The median follow-up period to BDS diagnosis was 45.5 months (range, 5–84) after LDLT. Univariate analysis revealed male sex, right lobe graft and bile duct strictures as factors that significantly correlated with BDS formation. Multivariate analysis revealed bile duct strictures (odds ratio, 7.17; $P = 0.011$) and right lobe graft (odds ratio, 10.20; $P = 0.040$) to be independent risk factors for BDS formation. One patient with BDS and biliary strictures succumbed to sepsis from cholangitis.

*Conclusion:* In the present study, right lobe graft and bile duct strictures are independent risk factors for BDS formation after LDLT. More careful observation and monitoring are required in the patients with high-risk factors.

**Key words:** bile duct stone, complication, living donor liver transplantation, male sex, right lobe graft

## INTRODUCTION

LIVER TRANSPLANTATION (LT) is a powerful therapy for patients with severe liver diseases, and its importance has been clearly recognized worldwide with the progression of surgical and perioperative care techniques. However, various complications still occur after LT, with biliary complications being relatively common. The reported incidence of biliary complications is approximately 5–25%.[1–3] Bile duct stone (BDS) is one of these biliary complications, often leading to severe cholangitis.[2,4,5] The reported incidence of BDS following LT is approximately 5%.[6,7] Moreover, several authors have reported the following risk factors for BDS after deceased donor LT: bile duct strictures, prolonged warm ischemia periods of grafts and increased total cholesterol levels.[8–11] However, few studies have analyzed BDS incidence after living donor liver transplantation (LDLT). In Japan, LDLT is predominantly performed because of the lack of deceased donor organs. This study aimed to review the clinical characteristics and outcomes of patients who developed BDS after LDLT and clarify the incidence of and risk factors for BDS after LDLT.

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## METHODS

WE ENROLLED 100 patients from a total of 157 patients who underwent LDLT at Nagasaki University Hospital from August 2000 to May 2012, excluding pediatric patients (aged <18 years) and patients who died in the early postoperative period (until 30 days). All of them were followed up for at least 5 months. We retrospectively reviewed their clinical course records, operative logs, blood examination, and radiology and endoscopic findings to analyze their clinical characteristics and risk factors for BDS.

In our institute, biliary reconstruction was performed by duct-to-duct anastomosis with an interrupting suture over a retrograde transhepatic biliary drainage tube (tube diameter, 2 mm) whenever possible. However, for patients with biliary atresia, primary sclerosing cholangitis and intraoperative bile duct injuries we selected hepaticojejunostomy over internal stenting. To evaluate the association between biliary ischemic change and BDS formation, the total ischemia time (TIT), defined as the duration from clamping of donor vessels to reperfusion of the recipients' portal vein, was also recorded. In our institute, periodic examinations are regularly conducted after transplantation.

We usually check the liver function of patients by blood examination once a month and perform abdominal enhanced computed tomography (CT) every 6 months, even if recipients have no symptoms. When we detected clinically suspicious symptoms of cholangitis such as abdominal pain with fever or abnormal increase in liver enzymes, we performed either magnetic resonance cholangiopancreatography (MRCP) or endoscopic retrograde cholangiography (ERC). When the cholangitis required drainage, our first choice was endoscopic treatment; therefore, percutaneous transhepatic cholangiography (PTC) was performed if ERC, including deep endoscopic procedures, failed because of bile duct deformity or hepaticojejunostomy. Bile duct stricture was defined as any narrowing of bile ducts identified by CT, MRCP or ERC that is associated with graft dysfunction and required any kind of interventional procedures. Hepatic artery complications and portal vein complications were diagnosed by enhanced CT and Doppler sonography.

Primary immunosuppression was induced after LDLT using standard dual therapy with tacrolimus (Tac) or cyclosporin (CyA) and steroids, although some patients with impaired renal function received basiliximab (BX) or mycophenolate mofetil (MMF).

### Statistical analysis

Categorical variables were analyzed using the $\chi^2$-test or Fisher's exact test, while continuous variables were analyzed using Student's *t*-test for normally distributed variables and the Mann–Whitney *U*-test for non-normally distributed variables. Logistic regression analysis was used to identify variables that independently predicted BDS incidence. A *P*-value of less than 0.05 was considered statistically significant in all analyses. All statistical analyses were performed using STATFLEX version 6 (Artech, Osaka, Japan).

## RESULTS

### Clinical characteristics of patients

IN TOTAL, 100 patients (42 men, 58 women; mean age, 52.9 ± 12.2 years [range, 22–72]) with LDLT were analyzed. The median observation period was 49.5 months (range, 5–143). The indications for liver transplantation are summarized in Table 1. The ABO blood type was incompatible in 15 (15%) patients. Of the 100 patients, 52 (52%) underwent right lobe transplantation and 48 (48%) underwent left lobe transplantation. For 92 (92%) patients, duct-to-duct anastomosis was selected to reconstruct the biliary system, while hepaticojejunostomy was selected for eight (8%). Multiple biliary reconstruction was performed in 13 patients, 12 of whom underwent right lobe grafting. Of the 13 patients, 12, including one who underwent left lobe grafting, required double anastomosis; the remaining one required triple anastomosis. The median TIT was 170 min (range, 106–555). Primary immunosuppression was induced after transplantation using Tac in 55 patients, Tac with MMF in 28 patients, CyA in nine, CyA with MMF in three, BX in one, BX with MMF in three and BX with Tac in one. With regard to other medications, ursodeoxycholic acid (UDCA) was used in 37 (37%) patients.

### Incidence of BDS and other complications

Ten patients (10%) developed BDS during the observation period. Of the 10 patients, four developed BDS in the proximal bile duct above the anastomotic site, including intrahepatic duct. Composition of the stones

**Table 1** Indications for liver transplantation

| Primary disease | No. of patients |
|---|---|
| Hepatitis B virus-related cirrhosis (LCB) | 28 |
| Hepatocellular carcinoma (HCC) in LCB | 13 |
| Hepatitis C virus-related cirrhosis (LCC) | 40 |
| HCC in LCC | 13 |
| LCC with hepatitis B virus | 2 |
| Alcohol-induced cirrhosis (LCAL) | 11 |
| HCC in LCAL | 3 |
| Non-alcoholic steatohepatitis (NASH) | 8 |
| HCC in NASH | 1 |
| Primary biliary cirrhosis | 4 |
| Primary sclerosing cholangitis | 1 |
| Fulminant hepatitis | 6 |
| Biliary atresia | 1 |
| Caroli disease | 1 |

was identified in six of 10 patients: one patients had a cholesterol stone and the rest had bilirubinate calcium stones. There was no bile duct filing defects diagnosed as biliary cast. The median duration from transplantation to BDS diagnosis was 45.5 months (range, 5–84). Twenty-two patients (14% of 157 patients) had bile duct strictures, six of whom also developed BDS. Bile duct stenting was performed for all patients with strictures, with 16 undergoing endoscopic stenting and six undergoing percutaneous stenting. Hepatic artery complications occurred in seven patients (7%): thrombosis ($n = 2$), endothelial dissection ($n = 1$), hemorrhage ($n = 2$) and blood flow decrease ($n = 2$). Patients with thrombosis, endothelial dissection and hemorrhage required surgical therapy, while blood flow decrease was treated with warfarin sodium.

## Risk factors for BDS formation

To clarify the risk factors for BDS formation, we analyzed the relationships among some clinical variables and BDS formation (Table 2). BDS was significantly common in male patients ($P < 0.05$), right lobe graft cases ($P < 0.05$) and those with bile duct strictures ($P < 0.01$). There was no significant difference in age, body mass index, Model for End-Stage Liver Disease score, rate of ABO blood type incompatibility, biliary reconstruction method (duct-to-duct anastomosis vs hepaticojejunostomy, single anastomosis vs multiple anastomosis), hepatic artery complications and TIT between patients with BDS and those without. We also analyzed whether serum total cholesterol and serum triglyceride levels were elevated above 200 mg/dL and 150 mg/dL, respectively, during the observation period; however, there were no significant differences between groups. With regarding to medication use, we found that the use of CyA and UDCA did not influence BDS formation.

Univariate analysis revealed that male sex, right lobe graft and bile duct strictures significantly correlated with BDS formation. Multivariate analysis revealed that bile

**Table 2** Multiple variables in living donor liver transplant patients with or without BDS ($n = 100$)

| Variables | BDS (+) | BDS (−) | *P* |
|---|---|---|---|
| Age (mean ± SD) | 58.3 ± 6.8 | 52.5 ± 12.6 | 0.146 |
| Sex (*n*) | | | 0.025 |
| Male | 9 | 49 | |
| Female | 1 | 41 | |
| BMI (mean ± SD) | 25.1 ± 3.3 | 23.9 ± 3.8 | 0.381 |
| MELD score (mean ± SD) | 17.4 ± 10.4 | 14.5 ± 8.1 | 0.344 |
| Graft lobe (*n*) | | | 0.011 |
| Right | 9 | 42 | |
| Left | 1 | 48 | |
| Blood type compatibility (*n*) | | | 0.720 |
| Match and compatible | 9 | 76 | |
| Incompatible | 1 | 14 | |
| Reconstruction manner (*n*) | | | 0.599 |
| Duct-to-duct anastomosis | 10 | 82 | |
| Hepaticojejunostomy | 0 | 8 | |
| Multiple anastomosis (*n*) | 1 | 11 | 0.657 |
| Bile duct stricture (*n*) | 6 | 16 | 0.002 |
| Hepatic artery complications (number) | 1 | 6 | 0.533 |
| TIT (median, min) | 178 (104–345) | 169 (108–555) | 0.381 |
| Primary IS (*n*) | | | 0.687 |
| Cyclosporin | 3 | 9 | |
| Tacrolimus/others | 7 | 81 | |
| Use of UDCA (*n*) | 4 | 33 | 0.920 |
| TC elevation >200 mg/dL (*n*) | 6 | 54 | 0.734 |
| TG elevation >150 mg/dL (*n*) | 5 | 42 | 0.539 |

*P*-value for age and MELD score based on Student's *t*-test, and for TIT based on Mann–Whitney *U*-test; all others based on Fisher's exact test.

BDS, bile duct stone; IS, immunosuppressant; MELD, Model for End-Stage Liver Disease; SD, standard deviation; TC, total cholesterol; TG, total triglyceride; TIT, total ischemic time; UDCA, ursodeoxycholic acid.

**Table 3** Risk factors for bile duct stone formation after living donor liver transplantation: Multivariate analysis ($n = 100$)

| Variables | OR | CI | *P* |
|---|---|---|---|
| Male sex | 6.00 | 0.65–55.79 | 0.115 |
| Right lobe graft | 10.20 | 1.12–93.21 | 0.040 |
| Bile duct stricture | 7.17 | 1.58–32.60 | 0.011 |

*P*-value for all variables based on multiple logistic regression analysis.
CI, confidence interval; OR, odds ratio.

duct strictures (odds ratio [OR], 7.17; $P = 0.011$) and right lobe graft (OR, 10.20; $P = 0.040$) were independent risk factors for BDS formation (Table 3).

## Treatment of BDS and clinical outcome

Four (40%) patients with BDS who were asymptomatic and showed no abnormalities in liver function test were carefully followed. On the other hand, six (60%) patients required admission and interventional procedures such as ERC and/or PTC because of cholangitis. The median number of admissions and length of hospitalization (days) were 2.67 (range, 1–4) and 37.8 (range, 8–125) respectively, for these patients. The treatments administrated to these patients and their clinical outcomes are shown in Table 4. In five of six treated patients, primary stone extraction was successful, and stone clearance had been confirmed using balloon cholangiography and intraductal ultrasonography. However, two patients developed recurrence of BDS and one had a residual intrahepatic stone. The patient who had a residual intrahepatic stone received stenting across stricture and stone, which stabilized their condition (Fig. 1).

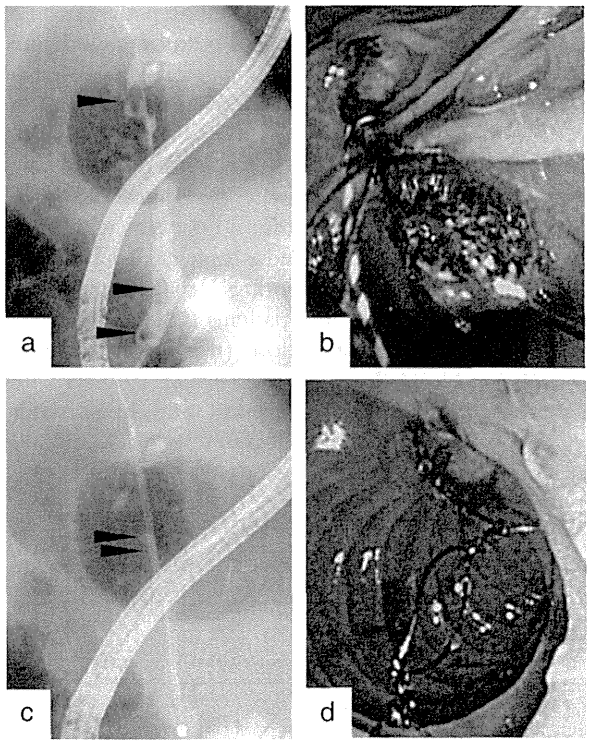


**Figure 1** (a) A patient with multiple bile duct stones (BDS; arrowhead), including intrahepatic (IH) stones, with anastomotic biliary strictures after living donor liver transplantation. (b) A stone located in the common bile duct was extracted successfully by using a basket catheter, and it was a calcium bilirubinate stone. (c) To prevent cholangitis caused by the residual IH stone, an internal stent (7-Fr, 5-cm, plastic stent) was inserted over the anastomotic stricture (arrowhead). (d) A 2-0 nylon thread was attached to the distal side hole of the stent for easy removal.

**Table 4** Summary of treatment and clinical outcome in bile duct stone cases

| Case (age, sex) | Location and size of BDS | Treatment | Clinical outcome |
|---|---|---|---|
| 63 years, F | CBD (10 mm) | These cases have been followed up with no symptoms | |
| 59 years, M | IH (13 mm) | | |
| 57 years, M | CBD (10 mm) | | |
| 65 years, M | CBD (5 mm) | | |
| 56 years, M | CBD, IH (multiple) | ESWL + PTC | Death of sepsis |
| 51 years, M | CBD (8.3 mm), IH (15.3 mm) | EST + stenting† | IH stone remained |
| 63 years, M | CBD (20 mm) | EPBD + stenting† | No recurrence |
| 54 years, M | CBD (5 mm) | EPBD + stenting† | No recurrence |
| 44 years, M | CBD (10 mm) | EST + stenting† | Recurrence in CBD |
| 66 years, M | IH (5 mm) | Stenting† | Recurrence in CBD |

†"Stenting" means internal tube-stent insertion over biliary stricture.
BDS, bile duct stone; CBD, common bile duct; EPBD, endoscopic papillary balloon dilation; EST, endoscopic sphincterotomy; ESWL, extracorporeal shock wave lithotripsy; IH, intrahepatic duct.

To prevent ascending cholangitis, we inserted stents into the bile duct in all the patients that required drainage. The stents were placed across the stricture, and the distal edge was located above the sphincter of Oddi. In all patients, we used the stent delivering system (Flexima Biliary Stent System; Boston Scientific, Marlborough, MA, USA) and modified plastic tube stent (sizes 7.0 Fr, a 2-0 nylon thread attached to the distal side-hole for easy removal). No procedure-related complications occurred in these patients.

One patient succumbed to sepsis following severe cholangitis. This patient was a 56-year-old man who underwent LDLT with duct-to-duct anastomosis using right lobe graft. Four month after LDLT, he developed biliary duct strictures with cholangitis; therefore, PTC and balloon dilation were performed because endoscopic therapy was impossible due to bile duct deformity. However, the patients developed repeated cholangitis, and all our attempt to clear BDS and bile duct strictures using non-surgical techniques, including cholangioscopy or extracorporeal shock wave lithotripsy, failed. Although the necessity of retransplantation was recognized and the procedure was scheduled, it was not undertaken because the patients developed sepsis with acute respiratory distress syndrome.

## DISCUSSION

IN OUR STUDY, BDS were developed in 10% of adult recipients who underwent LDLT. The reported incidence of post-transplant BDS varies widely among different study groups depending on the nature of the study population and manner of subject setting. In the study of Spier *et al.*, 49 of 1289 recipients (3.8%) developed BDS.[8] In the majority of the other studies, the incidence was reported to be approximately 5%,[6,7] whereas it was as high as 37% in another report.[12] In almost all previous studies, BDS was identified and diagnosed in patients who underwent examinations for clinically suspected cholangitis. However, some patients with BDS in our study had no symptoms and were incidentally diagnosed by protocol CT. Therefore, the incidence of BDS in the present study may be relatively higher than that in other reports, and our data may represent the actual state of BDS after LDLT.

According to multivariate analysis, bile duct strictures and right lobe graft were independent risk factors for BDS. The association between bile duct strictures and BDS has also been reported in previous studies.[8,10,11] We also speculate that bile duct strictures are likely to cause bile stasis and secondary infection, which results in the formation of bile duct sludge and stones. Nevertheless, eight patients developed common bile duct (CBD) stones, and of these, six had only CBD stones (Table 4). In addition, bile duct strictures were not observed in four of six patients with CBD stones. For this reason, it is suggested that some factors related to operation other than bile duct strictures, such as ischemic change or nerve disorder of the tissue surrounding CBD followed by biliary epithelial damage, influence BDS formation.

As shown above, right lobe graft was an independent risk factor of BDS. Some authors also indicated that the incidence of biliary complications was higher in patients who underwent LDLT with right lobe grafting than in those who underwent LDLT with left lobe grafting. In recent studies, the incidence of bile duct strictures in patients who underwent right lobe grafting was 8.3–32.8%,[13–15] while that in patients who underwent left lobe grafting was less than 15%.[16–18] We performed subgroup analysis to elucidate difference between patients who underwent right lobe grafting and those who underwent left lobe grafting and found no statistically significant difference in the incidence of bile duct strictures (Table 5). However, the number of men was significantly higher among the patients who underwent right lobe grafting. It is reasonable that the right lobe is selected to ensure appropriate size of grafts in male patients. With regard to the epidemiological survey of 1997 conducted by the Japanese Ministry of Health, Labor and Welfare, BDS was more common among males than among females. Although its cause is not clear, sex may have some relation to the development of BDS in patients who undergo right lobe grafting.

Several studies have reported that biliary ischemic change was a risk factor for the development of BDS after LDLT.[6,10] In patients with biliary cast syndrome in particular, identified as the hard, dark material taking the physical shape of the bile duct, biliary ischemia is believed to damage the bile duct mucosa and lead to cast formation.[10] However, ischemic factors such as TIT or hepatic artery complications were not detected as significant risk factors for BDS in the present study. We suggest that the characteristics of patients without cast formation contribute to this result.

Recently, endoscopic treatment is usually chosen as the primary approach for the management of biliary complications following LT.[19–21] Endoscopic procedure also makes it possible to shorten hospitalization of most post-transplant BDS patients with less invasiveness.[8,11,22] However, in some difficult situations, such as displacement of duodenal papilla or deformity of biliary tract,

**Table 5** Comparison and univariate statistical analysis between right lobe graft and left lobe graft. ($n = 100$)

| Variables | Right lobe graft | Left lobe graft | *P* |
|---|---|---|---|
| Bile duct stone (*n*) | 9/52 | 1/48 | 0.011 |
| Age (mean, years) | 51.8 | 53.9 | n.s. |
| Sex (number, male/female) | 36/16 | 22/26 | 0.018 |
| MELD score (mean, points) | 15.1 | 14.7 | n.s. |
| ABO incompatibility (*n*) | 7/52 | 8/48 | n.s. |
| TIT (median, min) | 177 (104–555) | 165 (109–250) | n.s. |
| Hepatic artery complication (*n*) | 3/51 | 4/48 | n.s. |
| Bile duct stricture (*n*) | 13/51 | 9/48 | n.s. |
| Cholangitis (*n*) | 18/51 | 10/48 | n.s. |

*P*-value for age and MELD score based on Student's t-test, and for TIT based on Mann-Whitney *U*-test; all others based on Fisher's exact test.
MELD, Model for End-Stage Liver Disease; n.s., not significant; TIT, total ischemic time.

endoscopic intervention is somewhat complicated and challenging. In addition, duodenobiliary reflux and bacterial contamination of bile duct related to recurrence of BDS may occur after endoscopic intervention. Many authors reported that the incidence of biliary complications, such as cholangitis and recurrence of BDS, was higher in patients after EST than in those after endoscopic papillary balloon dilation (EPBD).[23–25] Moreover, Natsui *et al.* reported that EPBD has a possibility of suppressing bacterial contamination of the biliary tract compared with EST in patients with small stones.[26] Therefore, it is desirable to choose EPBD for treatment of BDS whenever possible, especially in patients treated with immunosuppressants after transplantation. Although we mainly treated patients who underwent right lobe grafting in the present study, it appears that there is no great difference between right lobe graft cases and left lobe graft cases regarding treatment of CBD stones. Nevertheless, in the case of BDS located in the proximal bile duct above the anastomotic site, endoscopic intervention may be more difficult in patients with right lobe grafting than in those with left lobe grafting because of multiple biliary reconstruction or acute angulation of the bile duct. As described in Table 4, we performed endoscopic therapy in six of 10 patients who developed BDS, with successful stone removal in five patients (83%). The success rate of stone extraction in previous studies ranged 71–100%.[8,11,22] We believe that endoscopic therapy for BDS can be successfully performed in most cases, even after transplantation. However, two patients (40%) developed recurrence of BDS in our study, and both also had biliary strictures. The recurrence rate of treated BDS developed after LT has rarely been reported. In the study of Rerknimitr *et al.*, eight of 46 patients (17%) developed recurrence of BDS after treatment.[2] In previous reports on not post-transplant populations, the BDS recurrence rate was 3.2–8.8%.[27–29] As mentioned above, biliary stricture is an independent risk factor, besides it can also be considered as a cause of high recurrence rate in the absence of drastic treatment, namely surgery, including retransplantation. One patient with biliary strictures and BDS in our study succumbed to biliary sepsis during the observation period. The optimal timing of retransplantation is difficult to determine because of the limited supply of organs available for LT. In Japan, the shortage of donors is a particularly serious problem because deceased organ donation is not well established owing to religious beliefs. Therefore, we have to rely on graft donation from family members in most patients. However, this is sometimes a restricting factor for retransplantation.

Several studies about post-transplantation BDS, including biliary cast syndrome, have been reported till date; however, none have centrally focused on BDS after LDLT. In the present study, we determined the risk factors for and clinical features and clinical outcomes of BDS following LDLT. We identified two independent risk factors, namely bile duct strictures and right lobe graft which were significantly related to BDS formation after LDLT. Furthermore, bile duct stricture may be a predictor of poor outcome in patients with BDS after LDLT. Therefore, we should pay special attention to LDLT patients who develop BDS accompanied by bile duct strictures and schedule timely retransplantation. We believe that it is important to shorten follow-up period of patients with bile duct stricture, especially in right lobe graft cases.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

# The morbidity and associated risk factors of cancer in chronic liver disease patients with diabetes mellitus: a multicenter field survey

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**Abstract**
*Background and aims* Diabetes mellitus is associated with various cancers; however, little is known of the relationship between cancer and diabetes in chronic liver disease (CLD) patients. The aim of this study is to investigate the morbidity and associated factors of cancer, including the use of anti-diabetics, in CLD patients with diabetes.
*Patients and methods* We performed a multicenter survey in 2012 and 478 CLD patients with diabetes were enrolled (age 64.3 ± 12.1 years, female/male 187/291). A frequency analysis of cancer and antidiabetic use was performed. Independent factors for cancer were analyzed using logistic regression and decision-tree analysis.
*Results* The morbidity of cancer was 33.3 %. Hepatocellular carcinoma (HCC) and extra-hepatic cancer were diagnosed in 24.7 and 11.3 % of enrolled patients, respectively. The frequency of antidiabetic use was 66.5 %. Of prescribed antidiabetics, 39 % were dipeptidyl-peptidase 4 inhibitors; however, their use was not significantly associated with cancer. In contrast, the use of exogenous insulin (OR 2.21; 95 % CI 1.16–4.21, $P = 0.0165$) and sulfonylurea (OR 2.08; 95 % CI 1.05–3.97, $P = 0.0353$) were independently associated with HCC and extra-hepatic cancer, respectively. In decision-tree analysis, exogenous insulin and sulfonylurea were also identified as a divergence factor for HCC and extra-hepatic cancer, respectively.
*Conclusions* We found a high morbidity of not only HCC, but also extra-hepatic cancer in CLD patients with diabetes. We also showed a possible association between the use of antidiabetics and the morbidity of cancer. Thus, a large-scale cohort study is needed to establish a therapeutic strategy for diabetes to suppress carcinogenesis in CLD patients.

**Keywords** Diabetes mellitus · Chronic liver disease · Morbidity · Risk factor

**Abbreviations**

| | |
|---|---|
| HCC | Hepatocellular carcinoma |
| CLD | Chronic liver disease |
| DPP-4 | Dipeptidyl peptidase-4 |
| AST | Aspartate aminotransferase |
| APRI | AST to platelet ratio index |
| ALT | Alanine aminotransferase |
| GGT | Gamma-glutamyl transpeptidase |
| HbA1c | Hemoglobin A1c |

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HCV Hepatitis C virus
HBV Hepatitis B virus
MAPK Mitogen-activated protein kinase
IGF Insulin-like growth factor

## Introduction

Diabetes mellitus is a known independent risk factor for a number of different cancers [1]. Recently, population-based studies and meta-analyses demonstrated that diabetes mellitus is a potent risk factor for hepatocellular carcinoma (HCC) [2, 3]. In addition, diabetes mellitus is a risk factor for extra-hepatic cancers including pancreatic cancer, bile duct cancer, and colon cancer [4–6], and is also known to increase the risk of other extra-hepatic cancers, including gynecologic cancers, respiratory tumors, and hematological malignancies [7–9].

Diabetes mellitus consists of a number of diverse diseases, including impaired insulin secretion and insulin resistance. Patients with chronic liver disease (CLD) often develop increased insulin resistance and pancreatic β cells consequently secrete excess insulin in order to maintain glucose homeostasis [10, 11]. Thus, hyperinsulinemia is a feature of CLD patients with diabetes. Insulin is a potent mitogen and promotes cell proliferation [12], and hyperinsulinemia is a risk factor for the development of cancer in patients with diabetes mellitus [1, 13]. These previous findings suggest a possible association between cancer and diabetes in CLD patients; however, no practical data are available for the morbidity of cancer in CLD patients with diabetes.

Established risk factors for carcinogenesis include age, sex, smoking, excessive alcohol intake, and chronic viral infection [14]. In addition, we, along with others, have reported a possible association between the use of anti-diabetic agents and carcinogenesis [15, 16]. The use of sulfonylurea, an insulin secretagogue, and exogenous insulin are associated with HCC and extra-hepatic cancers including pancreatic cancer, colon cancer, and breast cancer [15–18]. Recently, dipeptidyl peptidase-4 (DPP-4) inhibitor has become widely used to treat diabetes mellitus because of its ability to lower glucose levels with a low risk of hypoglycemia; however, a possible association between the use of DPP-4 inhibitors and cancer has never been investigated in CLD patients with diabetes.

The aims of this study were to investigate the morbidity of cancer and cancer-associated factors, including the use of anti-diabetics, in CLD patients with diabetes.

## Subjects and methods

### Ethics

The study protocol conformed to the ethical guidelines of the Declaration of Helsinki, as reflected in the prior approval given by each institutional review board. None of the subjects were institutionalized.

### Study design

In 2012, we performed a multicenter cross-sectional study to investigate the morbidity of cancer and cancer-associated factors, including the use of anti-diabetics, in CLD patients with diabetes.

### Subjects

Inclusion criteria were patients with (1) 20 years of age or more, (2) CLD complicated with diabetes mellitus, and (3) regular medical consultations with a hepatologist. Exclusion criteria were (1) type 1 diabetes mellitus, juvenile diabetes mellitus, or gestational diabetes mellitus, (2) severe pancreatitis, (3) adrenal gland disease, (4) pituitary disease, and (5) a gonadal disorder. We enrolled 478 CLD patients with diabetes in this study from five medical institutions in Japan.

### Definition of CLD and its etiology

Regardless of the etiology of liver disease, chronic liver disease was diagnosed on the basis of hepatic inflammation that had lasted for more than 6 months, and findings of histopathology, ultrasonography, computed tomography, or magnetic resonance imaging.

The etiology of CLD was examined by biochemical tests, imaging examinations, and/or liver biopsy as previously described [19–23]. Briefly, chronic hepatitis C was diagnosed by positive results of anti-hepatitis C virus (HCV) and/or HCV RNA [20]. Chronic hepatitis B was diagnosed by positive results of hepatitis B surface antigen and/or hepatitis B virus (HBV) DNA [20]. Autoimmune hepatitis was diagnosed by the Diagnostic Criteria of the International Autoimmune Hepatitis Group [21]. Primary biliary cirrhosis was diagnosed based on the Clinical Guideline of Primary Biliary Cirrhosis by the Intractable Hepato-Biliary Disease Study Group [22]. Non-alcoholic fatty liver disease was diagnosed based on the Practice Guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology [19]. Alcoholic liver disease was diagnosed according to the Diagnostic

Criteria of Alcoholic Liver Disease by the Japanese Society for Biomedical Research on Alcohol [23].

### Definition of liver cirrhosis

Liver cirrhosis was diagnosed by aspartate aminotransferase (AST) to platelet ratio index (APRI); serum AST level (U/L)/upper limit of normal AST (33 U/L) × 100/platelet count ($\times 10^6$/mL). APRI is a noninvasive index and can predict liver cirrhosis. Patients with APRI values above 2 were diagnosed as with liver cirrhosis as previously described [24].

### Definition of diabetes mellitus

Diabetes mellitus was diagnosed on the basis of fasting blood glucose levels >126 mg/dL or HbA1c levels >6.5 % according to the Diagnostic Criteria for Diabetes Mellitus [25], or by the use of anti-diabetic agents.

### Definition of cancer

Cancer was defined as any type of malignant neoplasm including epithelial and non-epithelial tumors. The diagnosis of cancer was based on finding(s) of histopathology and/or by a combination of serum tumor makers and imaging procedures such as ultrasonography, computed tomography, magnetic resonance imaging, endoscopy, and/or angiography.

### Diagnosis of HCC

HCC was diagnosed by a combination of tests for serum tumor makers such as alpha-fetoprotein and des-gamma-carboxy prothrombin, and imaging procedures such as ultrasonography, computed tomography, magnetic resonance imaging, and/or angiography.

### Definition of extra-hepatic cancer, digestive cancer, and non-digestive cancer

Extra-hepatic cancer was defined as cancer in any organ except for the liver, and was further classified as either digestive cancer or non-digestive cancer. Digestive cancer was defined as cancer in the oral cavity, esophagus, stomach, colon, gallbladder, or pancreas. Cancer other than digestive cancer was defined as non-digestive cancer. The diagnosis of each cancer was based on finding(s) of histopathology and/or by a combination of serum tumor makers and imaging procedures such as ultrasonography, computed tomography, magnetic resonance imaging, endoscopy, and/or angiography.

### Definition of cardiovascular event

A cardiovascular event was defined as acute myocardial infarction or stroke, the diagnosis of which was based on clinical symptoms and findings of electrocardiogram recordings, biochemical tests, echocardiography, coronary angiography, computed tomography, or magnetic resonance imaging as previously reported [26].

### Diagnosis of diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy

Diagnosis of diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy was based on findings of urine and biochemical tests, ophthalmoscopy, tendon reflex tests, and vibration sense tests as previously described [27–29].

### Database

Using on medical records, a database of 478 CLD patients with diabetes was created on the basis of the following six categories:

Category 1: age, sex, body mass index, and blood pressure.
Category 2: any type of cancer, HCC, extra-hepatic cancer, digestive cancer, and non-digestive cancer.
Category 3: cardiovascular disease, diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy.
Category 4: chronic hepatitis C, chronic hepatitis B, non-alcoholic fatty liver disease, alcoholic liver disease, autoimmune hepatitis, and primary biliary cirrhosis.
Category 5: platelet count, serum AST level, serum alanine aminotransferase (ALT) level, serum gamma-glutamyl transpeptidase (GGT) level, serum albumin level, serum total bilirubin level, prothrombin activity, serum total cholesterol level, and serum triglyceride level.
Category 6: disease duration of diabetes mellitus, fasting blood glucose level, blood hemoglobin A1c (HbA1c; National Glycohemoglobin Standardization Program; NGSP), and use of a DPP-4 inhibitor, sulfonylurea, exogenous insulin, α-glucosidase inhibitor, biguanide, glinide, thiazolidine, and glucagon-like peptide 1 agonist.

### Statistical analysis

Data are expressed as the number or mean ± standard deviation (SD). Nonparametric comparisons were made using the Wilcoxon signed-rank test, and categorical comparisons were made using Fisher's exact test. Independent factors for cancer were analyzed using logistic regression and decision-tree analysis as described

previously [30, 31]. The level of statistical significance was set at $P < 0.05$.

## Results

### Patient characteristics

The patient characteristics are summarized in Table 1. The mean age was 64.3 years and the ratio of women to men was 1:1.56. Chronic hepatitis C and non-alcoholic fatty liver disease were the major etiologies of chronic liver disease. Liver cirrhosis was seen in 14.9 % of enrolled patients. APRI values were significantly higher in patients with chronic hepatitis C, chronic hepatitis B, and alcoholic liver disease (Supplementary Table 1).

**Table 1** Patient characteristics

| | Subjects |
|---|---|
| *N* | 478 |
| Age (years) | 64.3 ± 12.1 |
| Sex (female/male) | 187/291 |
| Body mass index (kg/m$^2$) | 24.5 ± 4.2 |
| Systolic/diastolic blood pressure (mmHg) | 128.9 ± 12.4/ 74.6 ± 12.3 |
| Etiology of chronic liver disease | |
| Chronic hepatitis C | 38.1 % (182/478) |
| Chronic hepatitis C with sustained virologic response by interferon therapy | 8.6 % (41/478) |
| Chronic hepatitis B | 7.3 % (35/478) |
| Non-alcoholic fatty liver disease | 29.5 % (141/478) |
| Alcoholic liver disease | 6.9 % (33/478) |
| Autoimmune hepatitis | 5.4 % (26/478) |
| Primary biliary cirrhosis | 2.5 % (12/478) |
| Others | 1.7 % (8/478) |
| Biochemical examinations | |
| Platelet count ($\times 10^3$/mm$^3$) | 16.1 ± 7.4 |
| AST (IU/L) | 43.0 ± 30.6 |
| ALT (IU/L) | 42.2 ± 36.1 |
| GGT (IU/L) | 77.4 ± 122.8 |
| Albumin (g/dL) | 3.93 ± 0.58 |
| Prothrombin time (%) | 91.5 ± 20.2 |
| Total bilirubin (mg/dL) | 0.88 ± 0.42 |
| Total cholesterol (mg/dL) | 172.4 ± 38.4 |
| Triglyceride (mg/dL) | 129.2 ± 94.8 |
| Presence of liver cirrhosis | 14.9 % (71/407) |
| APRI | 1.11 ± 1.18 |

Data are expressed as number or mean ± SD

*AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *GGT* gamma-glutamyl transpeptidase, *APRI* AST to platelet ratio index

The variables associated with diabetes mellitus are summarized in Table 2. The mean HbA1c level was 6.5 %. The morbidity of diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy were 10.4, 12.1, and 5.8 %, respectively.

Overall, 66.5 % (318/478) patients were treated with an antidiabetic agent. DPP-4 inhibitor was the most frequently prescribed (39.0 %), followed by sulfonylurea (25.5 %) and exogenous insulin (25.5 %) (Table 2).

### The morbidity of cardiovascular disease

The morbidity of cardiovascular disease was 6.1 % (29/478) and there were no etiological differences in the morbidity of cardiovascular disease (Supplementary Table 2).

### The morbidity of cancer

The morbidity of cancer was 33.3 % (159/478). Among the patients with cancer, multiple primary tumors were found in 10.0 % of cases (9.4 and 0.6 % for double and triple cancer, respectively). The overall morbidity of HCC was 24.7 % (118/478) (Fig. 1a) and patients with chronic hepatitis C, chronic hepatitis B, and alcoholic liver disease showed significantly higher morbidity of HCC (Supplementary Table 2).

The morbidity of extra-hepatic cancer was 11.3 % (54/478) (Fig. 1a). Amongst the patients with extra-hepatic cancer, digestive cancer and non-digestive cancer

**Table 2** Glucose metabolism, complications of diabetes, and use of anti-diabetic medication

| | Subjects ($n = 478$) |
|---|---|
| Disease duration of diabetes mellitus (year) | 5.4 ± 5.6 |
| Fasting blood glucose (mg/dL) | 135.7 ± 44.4 |
| HbA1c (%) | 6.5 ± 0.9 |
| Diabetic retinopathy | 10.4 % (48/478) |
| Diabetic nephropathy | 12.1 % (56/478) |
| Diabetic neuropathy | 5.8 % (27/478) |
| Use of anti-diabetic agent | 66.5 % (318/478) |
| DPP-4 inhibitor | 39.0 % (124/318) |
| Sulfonylurea | 25.5 % (81/318) |
| Exogenous insulin | 25.5 % (81/318) |
| α-Glucosidase inhibitors | 23.9 % (76/318) |
| Metformin | 16.7 % (53/318) |
| Glinides | 8.8 % (28/318) |
| Pioglitazone | 6.6 % (21/318) |
| GLP-1 agonists | 5.3 % (17/318) |

Data are expressed as number or mean ± SD

*HbA1c* hemoglobin A1c, *DPP-4* dipeptidyl-peptidase 4, *GLP* glucagon-like peptide

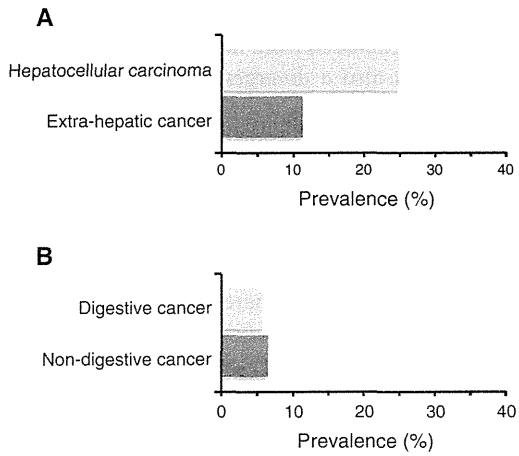


**Fig. 1** **a** The morbidity of hepatocellular carcinoma and extra-hepatic cancer. **b** The morbidity of digestive cancer and non-digestive cancer

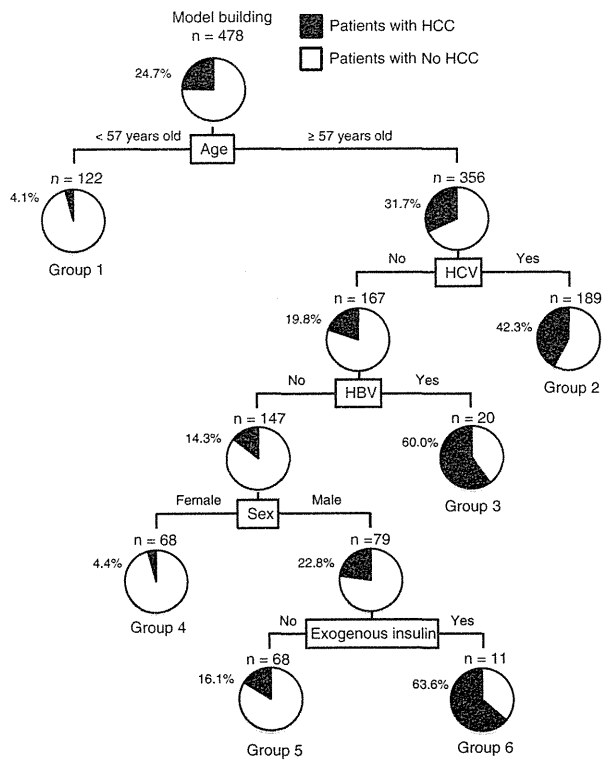


**Fig. 2** Decision-tree algorithm for hepatocellular carcinoma. The subjects were classified according to the indicated cutoff value for each variable. The *pie graphs* indicate the proportion of patients with no hepatocellular carcinoma (*white*) and patients with hepatocellular carcinoma (*black*) in each group. *HCC* hepatocellular carcinoma, *HCV* hepatitis C virus, *HBV* hepatitis B virus

accounted for 5.6 % (27/478) and 6.5 % (31/478) of cases, respectively (Fig. 2b). There were no etiological differences in the morbidity of extra-hepatic cancer, digestive cancer, and non-digestive cancer (Supplementary Table 2).

## Logistic regression analysis for cancer

In this analysis, non-alcoholic fatty liver disease, alcoholic liver disease, and APRI were not identified as independent factors associated with HCC. Age, chronic hepatitis C, chronic hepatitis B, and male gender were found to be independent risk factors for HCC (Table 3). Although HbA1c was not an independent risk factor, use of exogenous insulin was identified as an independent risk factor for the incidence of HCC (OR 2.21; 95 % CI 1.16–4.21; $P = 0.0165$) (Table 3). The use of sulfonylurea was identified as an independent risk factor for extra-hepatic cancer (OR 2.08; 95 % CI 1.05–3.97; $P = 0.0353$) (Table 3).

Even when patients with chronic hepatitis C and chronic hepatitis B were excluded from the analysis subjects, use of exogenous insulin or sulfonylurea was also identified as an independent risk factor for the incidence of HCC or extra-hepatic cancer, respectively (Supplementary Table 3 and 4).

## Decision-tree algorithm for HCC

In order to clarify the profile of HCC patients, a decision-tree algorithm was created using five divergence variables to classify six groups of patients (Fig. 2). An age of 57 years was the cutoff value for the initial classification. Among those patients aged ≥57 years, diagnosis of chronic hepatitis C was the variable for the second division. Among the patients with no hepatitis C virus (HCV) infection, diagnosis of chronic hepatitis B was the third division, and

**Table 3** Logistic regression analysis for the incidence of HCC and extra-hepatic cancer

| Event | Factors | Unit | Logistic regression analysis | | |
|---|---|---|---|---|---|
| | | | Odds ratio | 95 % confidence interval | *P* value |
| HCC | Age | 1 | 1.12 | 1.08–1.15 | <0.0001 |
| | Chronic hepatitis C | N/A | 5.20 | 2.88–9.81 | <0.0001 |
| | Chronic hepatitis B | N/A | 10.26 | 3.98–27.6 | <0.0001 |
| | Male | N/A | 2.50 | 1.44–4.45 | 0.0010 |
| | Use of exogenous insulin | N/A | 2.21 | 1.16–4.21 | 0.0165 |
| | HbA1c | 1 | 0.82 | 0.60–1.11 | 0.2046 |
| | GGT | 1 | 1.00 | 0.999–1.002 | 0.2009 |
| Extra-hepatic cancer | Age | 1 | 1.04 | 1.02–1.07 | 0.0008 |
| | Sulfonylurea | N/A | 2.08 | 1.05–3.97 | 0.0353 |
| | Chronic hepatitis C | N/A | 0.56 | 0.30–1.00 | 0.0532 |

*GGT* gamma-glutamyl transpeptidase, *HbA1c* hemoglobin A1c

among the patients with no hepatitis B virus (HBV) infection, gender was the fourth division. Then, among male patients, the use of exogenous insulin was the fifth division. Thus, 63.6 % of patients had HCC from among those who were aged ≥57 years, had no HCV or HBV infection, were male, and used exogenous insulin (Group 6; Fig. 2). On the other hand, 16.1 % of the patients not treated using exogenous insulin had HCC (Group 5; Fig. 2). In this analysis, non-alcoholic fatty liver disease or alcoholic liver disease was not identified a divergence variable for HCC.

Even when patients with chronic hepatitis C and chronic hepatitis B were excluded from the analysis subjects, use of exogenous insulin was also a divergence variable for the incidence of HCC (Supplementary Figure 1A and B).

### Decision-tree algorithm for extra-hepatic cancer

In order to clarify the profile of extra-hepatic cancer patients, a decision-tree algorithm was created using two divergence variables to classify three groups of patients (Fig. 3). An age of 78 years was the cutoff value for the initial classification. Among the patients who were aged ≥78 years, the use of sulfonylurea was the second division. Thus, 56.2 % of the patients aged ≥78 years and treated with sulfonylurea had extra-hepatic cancer (Group 3; Fig. 3). On the other hand, 21.3 % of the patients who were not treated with sulfonylurea had extra-hepatic cancer (Group 2; Fig. 3). Although it was not statistically significant, a tendency of high incidence of digestive cancer was seen in the sulfonylurea group compared to the non-sulfonylurea group in patients with extra-hepatic cancer (68.8 vs. 42.1 % $P = 0.0738$).

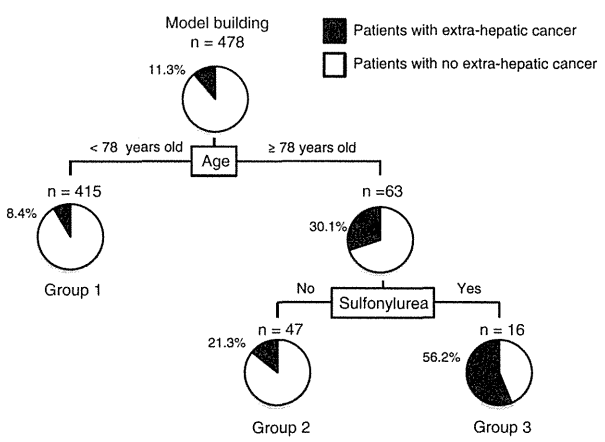


**Fig. 3** Decision-tree algorithm for extra-hepatic cancer. The subjects were classified according to the indicated cutoff value for each variable. The *pie graphs* indicate the proportion of patients with no extra-hepatic cancer (*white*) or patients with extra-hepatic cancer (*black*) in each group

Even when patients with chronic hepatitis C and chronic hepatitis B were excluded from the analysis subjects, use of sulfonylurea was also a divergence variable for the incidence of extra-hepatic cancer (Supplementary Figure 2A and B).

## Discussion

In this study, we found that there was a high morbidity of both HCC and extra-hepatic cancer in CLD patients with diabetes. Moreover, the use of sulfonylurea and exogenous insulin was independently associated with the risk of HCC and extra-hepatic cancer, respectively.

The morbidity of cardiovascular disease was 6.1 % in this study. In contrast, Limori et al. reported that the morbidity of cardiovascular disease was 26.8 % in diabetic patients [32]. It is unclear why there is a difference in the morbidity for cardiovascular disease between this previous study and our study; however, a possible explanation is the difference in etiology of diabetes mellitus. In this study, we enrolled CLD patients with diabetes. Serum cholesterol level is associated with atherosclerosis and subsequent microvascular and macrovascular events [33]. Since cholesterol synthesis is impaired in patients with chronic liver disease, the morbidity of cardiovascular disease may be relatively low in CLD patients with diabetes. In fact, the average level of cholesterol was in the normal range in this study and the morbidities of microvascular complications such as diabetic retinopathy, nephropathy, and neuropathy were lower than previously reported [34–36]. These findings support our hypothesis and suggest that a low morbidity of cardiovascular disease may be a feature of CLD patients with diabetes.

In this study, the morbidity of HCC was 24.7 %. This study was conducted in center hospitals for liver disease, and therefore, institutional bias may partly explain this finding. We also note that there was a high morbidity of extra-hepatic cancer. There are generally more opportunities to coincidentally detect digestive cancer in patients with chronic liver disease, as they are frequently examined by abdominal computed tomography and upper gastrointestinal endoscopy. In addition, we revealed that the morbidity of digestive cancer was similar to that of non-digestive cancer, indicating that carcinogenic potential may be higher in CLD patients with diabetes. An increased insulin resistance and subsequent hyperinsulinemia is a hallmark of CLD patients with diabetes [10, 11]. Insulin binds to the insulin receptor and activates the insulin receptor substrate/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase cascade [13]. Insulin can also bind to the insulin-like growth factor (IGF)-1 receptor and subsequently activate the Raf/MAPK

kinase/MAPK pathway [37]. Moreover, excess insulin competes with IGF-1 binding to the IGF-binding protein, resulting in an increase in the serum level of IGF-1, which is a potent stimulator of carcinogenesis [13]. Taken together, hyperinsulinemia may be one of the causes of the high morbidity of cancer in CLD patients with diabetes.

The possible relationship between the use of antidiabetic agents and carcinogenesis remains a controversial issue. In this study, we found that DPP-4 inhibitor was the most frequently prescribed agent, accounting for 39.0 % of all prescribed antidiabetic agents. Although we could not evaluate the period of antidiabetic mediation and this study was not designed to investigate a relationship between use of anti-diabetic agents and carcinogenesis, Kissow et al. [38] reported that DPP-4 inhibition did not accelerate neoplasia in carcinogen-treated mice, and White et al. [39] reported that the incidence of cancer was similar with DPP-4 inhibitor and placebo in diabetic patients with acute coronary syndrome. Likewise, in this study, we found that the use of DPP-4 inhibitor was not an independent risk factor for cancer in CLD patients with diabetes. However, logistic regression analysis revealed that the use of exogenous insulin and sulfonylurea was an independent risk factor for HCC and extra-hepatic cancer, respectively. Similar findings were also seen, even when patients with chronic hepatitis C and chronic hepatitis B were excluded from the analysis subjects. Both exogenous insulin and sulfonylurea increase the serum insulin level, which in turn could up-regulate mitosis and cell growth [12]. We and other groups have previously demonstrated that the use of exogenous insulin is a risk factor for HCC [15, 40], and sulfonylurea has been shown to be a risk factor for extra-hepatic cancers such as colon cancer [41] and pancreatic cancer [17]. Thus, the results of this study concur with those of previous reports.

Finally, we performed a decision-tree analysis, revealing that age was the first divergence factor for the incidence of both HCC and non-hepatic cancer. These findings indicate that aging is the most significant carcinogenic factor for cancer. In the decision-tree analysis, liver function tests and APRI were not divergence variables for the incidence of HCC. Since only 14.9 % of the enrolled patients were liver cirrhosis in this study, liver cirrhosis might not be selected as a factor associated with the incidence of HCC because of insufficient number of cirrhotic patients. In the algorithm for HCC, the use of exogenous insulin was not a significant risk factor in HCV-infected or HBV-infected patients, suggesting that HCV or HBV infection may dilute the impact of exogenous insulin on HCC. However, the use of exogenous insulin was a significant risk factor for male patients. Similarly, the use of exogenous insulin was a divergence variable for male patients, even when patients with chronic hepatitis C and chronic hepatitis B were excluded from the analysis subjects. IGF-1 is known to stimulate androgen receptor activity, through a β integrin-dependent mechanism, which also plays an important role in cancer progression [42] and might explain this gender-specific component of cancer risk.

For extra-hepatic cancer, sulfonylurea rather than exogenous insulin was found to be the second most significant risk factor. Similarly, the use of sulfonylurea was the second most significant risk factor for extra-hepatic cancer, even when patients with chronic hepatitis C and chronic hepatitis B were excluded from the analysis subjects. Sulfonylurea administration results in the increased expression of ATP-sensitive potassium channels, which in turn promotes insulin secretion from pancreatic beta cells as well as proliferation of various types of cancer cells in culture [43, 44]. Thus, in addition to a hyperinsulinemia-dependent mechanism, sulfonylurea may also directly increase the carcinogenic potential in very elderly patients. The association of anti-diabetic agents with cancer may therefore differ with the presence of other carcinogenic factor(s).

A limitation of this study is that we could not evaluate the precise duration of anti-diabetic medication. To investigate causal relationship between the use of anti-diabetic agents and carcinogenesis, further study will be focused on the duration of anti-diabetic medication.

In conclusion, in this study, we found that there is a high morbidity of both HCC and extra-hepatic cancer in CLD patients with diabetes. This study also revealed that the use of sulfonylurea and exogenous insulin were risk factors for HCC and extra-hepatic cancer, respectively, in CLD patients with diabetes. Thus, a large-scale cohort study is needed to identify therapeutic strategies for diabetes to suppress carcinogenesis in CLD patients.

**Conflict of interest** The authors declare that they have no conflict of interest.

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***Original Article***

# Significance of miRNA-122 in chronic hepatitis C patients with serotype 1 on interferon therapy

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*Aim:* Peginterferon (PEG IFN) and ribavirin combination therapy is a curative treatment for chronic hepatitis C virus (HCV) infection, and virological response to IFN therapy has been strongly associated with genetic variation in *IL28B* single nucleotide polymorphisms (SNP). Recently, miRNA122 (miR-122), which is the most abundant miRNA in the liver, has been reported to be important for the replication of HCV RNA. Therefore, we investigated the correlation of miR-122 expression with virological response to IFN and other clinical data.

*Methods:* A total of 51 patients with HCV infection who were treated with IFN therapy at Nagasaki University Hospital from 2006 to 2011 were included in this study. We investigated the correlation of miR-122 expression in liver biopsy specimens with virological response to IFN therapy and other predictors of response, including IL28 SNP.

*Results:* miR-122 expression did not correlate with IL28 SNP. However, a significant difference was observed in miR-122 expression between patients who showed a sustained virological response (SVR) and those who did not ($P < 0.05$). Multivariate analysis indicated that miR-122 is an independent predictor of SVR.

*Conclusion:* miR-122 expression could be a marker for predicting the outcome of IFN therapy. Therapies targeting miR-122 may have positive effects not only by directly inhibiting viral propagation but also by ameliorating cholesterol and lipid abnormalities.

**Key words:** chronic hepatitis C, interferon, miRNA-122

## INTRODUCTION

HEPATITIS C VIRUS (HCV) is a positive-strand RNA virus that has infected 170 million people worldwide. Once infected, 70–80% of patients experience persistent infection leading to repeated division of hepatocytes and ultimately fibrosis, cirrhosis and occasionally progression to hepatocellular carcinoma.[1,2] Therefore, the development of effective antiviral therapies against HCV is important. Treatment of chronic hepatitis C (CHC) infection has progressed from interferon (IFN) monotherapy to combination therapy with peginterferon (PEG IFN) and ribavirin (RBV), and direct-acting agents (DAA).[3–7] Such advances have improved the rate of sustained virological response (SVR) from 10% to 80% among CHC genotype 1 patients. Development of novel DAA is expected to further improve the prognosis of CHC patients. However, because of their severe adverse effects, not all patients can adapt to DAA. In the near future, we hope to be able to use newly developed DAA that do not have such severe side-effects. But such drugs have a problem for drug-resistance and possibility of viral mutation. So, despite the low SVR, combination therapy with PEG IFN and RBV may remain the one means of CHC treatment. To increase the cure rate as much as possible, factors capable of predicting SVR to CHC treatment should be identified. Numerous virus- and host-related factors are known predictors of SVR.[8–13] In 2009, a single nucleotide polymorphism

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(SNP) near *IL28B* was reported to be strongly associated with response to CHC treatment.[14]

miRNA are endogenous, small, non-coding RNA of approximately 21–22 nucleotides that have important gene regulatory functions in animals and plants; they bind to mRNA of protein-coding genes to direct their post-transcriptional repression.[15–17] miRNA are implicated in numerous biological processes and diseases, including viral infections and cancers.[18] They typically mediate their regulation by inducing mRNA destabilization or translational repression by binding to complementary sequences in the 3′-untranslated region (3′-UTR) of target mRNA.

miR-122 is a highly abundant, liver-specific miRNA that constitutes 70% of the total liver miRNA content. It positively modulates replication,[19] translation and virion production[20,21] by HCV by binding to the complementary target sequences in the 5′-UTR of HCV RNA.[19,22,23] Furthermore, sequestration of miR-122 in hepatoma cells by antisense oligonucleotides has been shown to decrease HCV replication and translation.[24,25]

Numerous *in vitro* studies have been performed, but few have examined the correlation of miR-122 with response to IFN therapy. A recent study of 42 patients who were seropositive for HCV RNA found that miR-122 was decreased in the livers of HCV patients, and that miR-122 correlated with clinical response to PEG IFN-α but not the HCV load.[26] We investigated whether miR-122 is a contributing factor to IFN treatment of CHC patients, and determined the correlation of miR-122 expression with virological response to IFN and other clinical parameters.

## METHODS

### Patients and clinical samples

CLINICAL DATA COLLECTED from the patients are listed in Table 1. Our study included 51 consecutive CHC patients who were treated with IFN therapy at Nagasaki University Hospital from January 2006 to April 2011. Forty-six patients in this study received PEG IFN-α-2b and RBV combination therapy, while five patients underwent PEG-IFN-α-2a and RBV combination therapy. Treatment duration ranged 48–72 weeks.

Twenty-two patients (43%) reduced IFN dose by neutropenia, and 21 patients (41%) reduced RBV dose by hemolytic anemia. Every case could continue IFN and RBV therapy. Adherence to IFN ranged 42–170% (mean, 98%) and adherence to RBV ranged 35–168% (mean, 98%). Twenty-one patients (41%) could achieve IFN adherence over 80%. Forty-eight patients (94%) could achieve RBV adherence over 60%.

**Table 1** Clinical characteristics

| Characteristics | Mean, number | Standard deviation (SD) |
|---|---|---|
| Age (years) | 57.4 | 9.26 |
| Sex (F/M) | 20/31 | |
| BMI (kg/m$^2$) | 23.2 | 3.42 |
| WBC (cells/μL) | 4762 | 1292 |
| PLT (×10$^4$ platelets/μL) | 17.41 | 5.765 |
| AST (IU/L) | 56.09 | 32.70 |
| ALT (IU/L) | 80.27 | 58.11 |
| γ-GT (IU/L) | 52.51 | 52.57 |
| Albumin (g/dL) | 4.102 | 0.294 |
| TC (mg/dL) | 174.08 | 23.94 |
| TG (mg/dL) | 100.68 | 38.92 |
| LDL-C (mg/dL) | 96.43 | 21.10 |
| HDL-C (mg/dL) | 51.64 | 12.45 |
| FFA (mEq/L) | 0.438 | 0.409 |
| PreAlb (mg/dL) | 19.52 | 4.708 |
| HbA1c (%) | 5.409 | 0.948 |
| HCV RNA (logIU/mL) | 6.2 | 1.15 |
| Staging (F0/1/2/3/4) | 8/18/12/8/4 | |
| Activity (A0/1/2) | 2/37/7 | |
| IFN adherence (%) | 98 (42–170) | |
| IFN adherence, >80% | 21 (41%) | |
| RBV adherence (%) | 98 (35–168) | |
| RBV adherence, >60% | 48 (94%) | |
| IFN response (SVR/TVR/NR) | 29/12/10 | |
| IL28B rs8099917 (TT/TG/GG) | 38/13/0 | |

Normal values in laboratory tests: body mass index (BMI) calculated as bodyweight (kg)/height (m)$^2$; white blood cell count (WBC, cells/μL), 3500–9000; platelets (PLT, ×10$^4$ platelets/μL), 12–33; aspartate aminotransferase (AST, IU/L), 10–40; alanine aminotransferase (ALT, IU/L), 5–40; γ-glutamyltransferase (γ-GT, IU/L), <70 in males, <30 in females; albumin (Alb, g/dL), 4.0–5.0; total cholesterol (TC, mg/dL), 128–220; triglyceride (TG, mg/dL), 38–150; low-density lipoprotein cholesterol (LDL-C, mg/dL), 70–139; high-density lipoprotein cholesterol (HDL-C, mg/dL), 40–80; free fatty acid (FFA, mEq/L), 100–800; pre-albumin (preAlb), 22–40; hemoglobin A1c (HbA1c), <5.8%.
HCV, hepatitis C virus; IFN, interferon; NR, undetectable HCV RNA; RBV, ribavirin; SD, standard deviation; SVR, sustained virological response.

The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Informed consent was obtained from each patient. Thereafter, a liver specimen was obtained by echo-guided liver biopsy. All liver biopsy tissue specimens were examined using hematoxylin–eosin, Azan–Mallory and silver reticulum staining. The specimens were assessed by one reviewer

blinded to patient clinical and biochemical data. Diagnosis of each case was independently and histologically confirmed by liver pathologists according to the Japanese chronic hepatitis classification (New Inuyama classification). Furthermore, we scored the degree of fat deposition in the liver. Histological characteristics of the patients are also shown in Table 1.

## Methods

### RNA isolation

Total RNA containing miRNA was isolated from formalin-fixed paraffin-embedded (FFPE) liver biopsy specimens using the RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Ambion, Austin, TX, USA) in accordance with the manufacturer's protocol.

### Quantitative reverse transcription polymerase chain reaction (PCR)

miR-122 expression was quantified using TaqMan MicroRNA assays (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's protocols. Reverse transcription was performed using 10 μg of RNA isolated from the liver FFPE specimens. Quantitative PCR was performed using the Light Cycler 480 system (Roche Diagnostics, Basel, Switzerland). miR-122 expression was calculated by the relative standard curve method and normalized to RNU6B expression. The *IL28B* SNP rs8099917 was also examined. SNP were detected by pyrosequencing analysis. The sense, antisense and pyrosequencing primers used were B-TCCTCCTTTTGTTTTCCTTTCTG, AAAAAGCCAGC TACCAAACTGT, and TGGTTCCAATTTGGG, respectively, where "B" indicates a biotin-labeled sequence.

## Statistical analysis

Data were processed on a personal computer and analyzed using StatFlex (Artech, Tokyo, Japan). Differences in each laboratory parameter were analyzed using the Mann–Whitney *U*-test. $P < 0.05$ was considered statistically significant.

# RESULTS

## Correlation of miR-122 expression with virological response to IFN therapy

WE COMPARED MIR-122 expression between *IL28B* rs8099917 TT and TG/GG. No significant difference was observed in hepatic miR-122 expression between *IL28B* SNP TT and TG/GG (Fig. 1a). We also compared miR-122 expression and stage of fibrosis. Although there was a tendency toward a decrease in miR-122 expression as fibrosis progressed, no statistically significant difference was detected (Fig. 1b). In terms of virological response to IFN therapy, a significant difference was observed in the extent of miR-122 expression and the number of patients who were classified as achieving an SVR or not ($P < 0.05$, Fig. 1c). We also investigated the correlation of miR-122 expression with SVR, transient response (TVR) and no response (NVR). Significant differences were observed between SVR and TVR ($P < 0.05$) and between SVR and NVR ($P < 0.05$) (Fig. 1d). As stated above, decrease of miR-122 was associated with progression of fibrosis. Therefore, we thought to compare the correlation of miR-122 expression to viral response to INF in patients matched for stage of fibrosis. Although we found that patients achieving SVR were likely to have higher expression of miR-122 than those who failed to achieve SVR, statistical difference was not observed due to limited patient numbers.

## Correlation of miR-122 expression with rapid/early virological response

miR-122 expression was significantly higher in patients with a strong response to IFN and undetectable HCV RNA at week 4 (i.e. those with a rapid virological response [RVR]) than in patients who did not achieve RVR (Fig. 2a, $P < 0.05$). Patients who responded to IFN therapy and who had undetectable HCV RNA at week 12 were regarded as achieving an early virological response (EVR). miR-122 expression showed the same tendency in these patients, namely, it was higher among those with EVR than among those without such a response (Fig. 2b, $P < 0.05$). No significant correlation was observed between miR-122 expression and several other clinical parameters including white blood cell count, red blood cell count, platelets, aspartate aminotransferase, alanine aminotransferase, γ-glutamyltransferase, albumin, pre-albumin, ferritin, type IV collagen and hyaluronic acid (Table 2).

## Fat deposition in the liver was associated with miR-122 expression

In addition, we scored the degree of fat deposition in liver biopsy specimens and examined its correlation with miR-122 expression. miR-122 expression significantly decreased as the extent of fat deposition in the liver increased (Fig. 3a, $P < 0.05$). We then divided the patient's biopsy specimens into groups based on the extent of fat deposition (0–5% and >5%) and determined their association with non-alcoholic fatty liver