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Original Article

Nutritional assessments for ordinary medical care in patients with chronic liver disease

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Aim: In patients with chronic liver disease who are at risk of malnutrition, simple and useful assessments for nutritional status should be established for ordinary medical care. The prognostic nutritional index (PNI) and controlling nutritional status (CONUT) are simple assessments constructed of only two or three laboratory data. We aimed to describe the potential of PNI and CONUT as a nutritional assessment tool in patients with chronic liver disease.

Methods: We enrolled 165 patients, aged 18–85 years, with chronic liver disease. These patients were nutritionally assessed by PNI or CONUT, demonstrating the association with the severity of chronic liver disease or anthropometric values.

Results: The value of PNI or CONUT was significantly associated with the severity of chronic liver disease ($P < 0.001$, respectively). In addition, the value of CONUT was significantly associated with all the anthropometric values such as body mass index (BMI, $P < 0.05$), mid-arm circumference (AC, $P < 0.001$), mid-arm muscle circumference (AMC, $P < 0.001$), and triceps skinfold thickness (TSF, $P < 0.001$), whereas the value of PNI was significantly associated with the values of AC ($P < 0.01$), AMC ($P < 0.05$) and TSF ($P < 0.05$). Approximately 80% of cirrhotic patients were assessed by PNI or CONUT to have obvious malnutrition.

Conclusion: PNI and CONUT are potential tools for nutritional assessment in patients with chronic liver disease, especially for ordinary medical care, because of their simplicity.

Key words: anthropometry, controlling nutritional status, liver cirrhosis, malnutrition, mid-arm muscle circumference, prognostic nutritional index, triceps skinfold thickness

INTRODUCTION

PROTEIN-ENERGY MALNUTRITION (PEM) is a life-threatening complication in patients with liver cirrhosis (LC).[1–7] Approximately 60–90% of patients with LC have been reported to suffer from PEM.[2,6,8,9]

Respiratory quotient (RQ) is one of the most sensitive and beneficial assessments for energy malnutrition.[10] Especially in cirrhotic patients, increase in fat oxidation as fuel after overnight fast has been well characterized, resulting in a decrease in non-protein RQ (npRQ).[6,11–17] Moreover, the prognosis in cirrhotic patients with npRQ of 0.85 or more has been demonstrated to be greater than in those with npRQ of less than 0.85.[6,7] However, the nutritional assessment by npRQ is not always available in a clinical setting because an indirect calorimeter is required for npRQ measurement. Therefore, simple assessments for nutritional status should be established.

One of the simple and standard assessments for nutritional status is subjective global assessment (SGA).[18] SGA assesses nutritional status based on simple questions and physical findings. However, SGA is not sufficient for nutritional assessment in patients with chronic liver disease because it underestimates nutritional status compared to assessments containing laboratory data.[8,19]

Another of the simple and standard assessments for nutritional status is anthropometry, such as body mass index (BMI), triceps skinfold thickness (TSF) and mid-arm muscle circumference (AMC).[18] However, similar to

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SGA, nutritional assessment by anthropometry is suggested to underestimate nutritional status in cirrhotic patients.[2,8]

The prognostic nutritional index (PNI) and controlling nutritional status (CONUT) are representatives for simple assessment of nutritional status. The PNI proposed by Onodera *et al.*[20] is a formula that is simply constructed from laboratory data such as albumin and total lymphocyte count (TLC). Nevertheless, the PNI has been demonstrated to predict the outcome in cancer patients.[20–24] CONUT was proposed by Ignacio de Ulíbarri *et al.* and is constructed from laboratory data such as albumin, TLC and total cholesterol (TC). CONUT has been shown to associate with other nutritional assessments.[25] Therefore, the PNI and CONUT could be candidates for nutritional assessment, with wide clinical application; however, there are no data available to demonstrate the usefulness of the PNI and CONUT as nutritional assessment tools in patients with chronic liver disease.

In this study, we aimed to describe the potential of the PNI and CONUT as nutritional assessment tools in patients with chronic liver disease, demonstrating the association between disease progression and nutritional status.

Table 1 Patient profiles

Age (years)	66.9 ± 0.7
Sex (F/M)	61/104
Hepatitis C virus	113
Hepatitis B virus	17
Alcohol abuse	42
Liver cirrhosis	138
Hepatocellular carcinoma	113
Body mass index	23.1 ± 0.3
Mid-arm muscle circumference (%)	96.9 ± 0.9
Triceps skinfold thickness (%)	102 ± 3.5
Total bilirubin (mg/dL)	1.79 ± 0.2
Aspartate aminotransferase (U/L)	66.4 ± 3.4
Alanine aminotransferase (U/L)	47.7 ± 2.8
Total protein (g/dL)	7.01 ± 0.1
Albumin (g/dL)	3.06 ± 0.1
Choline esterase (U/L)	115 ± 6.9
Total cholesterol (mg/dL)	133 ± 3.1
Triglyceride (mg/dL)	86.8 ± 3.9
Hemoglobin (g/dL)	11.5 ± 0.2
Total lymphocyte count (/μL)	1188 ± 47
Prothrombin time (international normalized ratio)	1.28 ± 0
Prognostic nutritional index	36.5 ± 0.6
Controlling nutritional status (score)	6.2 ± 0.2

METHODS

Subjects

WE IDENTIFIED 178 patients who were hospitalized in the Gastroenterological Unit of Kurume University Hospital from November 2005 to October 2010, for whom anthropometric data such as bodyweight, body height, TSF and mid-arm circumference (AC) could be obtained. Patients without chronic liver disease, and patients aged less than 18 years or 85 years or more were excluded. Finally, 165 patients were enrolled in this study. The etiology of chronic liver disease was hepatitis C virus ($n = 113$) and hepatitis B virus ($n = 17$) infection, and other causes ($n = 35$). Alcohol abuse (≥50 g/day) was found in 42 patients. Chronic liver disease developed to cirrhosis in 138 patients, and hepatocellular carcinoma was diagnosed in 113 patients. Liver function in cirrhotic patients was classified into three degrees of severity according to the Child–Pugh classification (class A, B and C). Liver function was finally categorized into four degrees of severity, chronic hepatitis (CH, $n = 27$) and LC class A, B and C ($n = 36$, 78 and 24, respectively). All diagnoses were based on clinical, serological, histological and/or imaging evidence. The patient profiles are shown in Table 1.

Informed consent was obtained from all subjects. This study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in prior approval by the Ethics Committee of Kurume University School of Medicine. No participant was institutionalized.

Anthropometric measurement

Anthropometric measurement included BMI, AC, TSF and AMC. TSF was measured by experienced observers with a caliper (Abbott Japan, Tokyo, Japan) at the middle point between the acromion and the olecranon of the non-dominant arm. AC was measured with a tape (Abbott Japan) at the same site of TSF. To minimize variability, TSF or AC was consecutively measured three times and each average was recorded. AMC was calculated by the following formula: AMC (cm) = AC (cm) – (0.314 × TSF [mm]). TSF and AMC were expressed as the percentage of sex- and age-matched mean values in the Japanese population.[26]

Laboratory data

Venous blood samples were taken in the morning after an 8-h overnight fast. Laboratory data were measured by conventional clinical methods (Department of Clinical Laboratory, Kurume University Hospital) as previously described.[27] All laboratory data in this study were collected from the data measured within 72 h of anthropometric measurement.

Nutritional assessment

Anthropometry, SGA, albumin, TLC, TC, PNI and CONUT were used for nutritional assessment. Nutritional status assessed by SGA was classified into two categories, no malnutrition or malnutrition, regardless of its severity. The PNI was described by Onodera *et al.*[20] and was calculated as follows: PNI = albumin × 10 + TLC × 0.005. Nutritional status assessed by the PNI was classified into four degrees of severity as follows: PNI of 50 or more, no malnutrition; 40–49, mild malnutrition; 30–39, moderate malnutrition; and less than 30, severe malnutrition. Nutritional status assessed by CONUT was classified into four degrees of severity (no malnutrition and mild, moderate and severe malnutrition) according to the original study of Ignacio de Ulíbarri *et al.* (shown in Table 2).[25]

Statistical analysis

Data were expressed as mean ± standard deviation. Non-parametric multiple comparisons among the groups were analyzed by the Kruskal–Wallis test, following the Dunn's multiple comparison tests to compare all pairs of groups. Categorical comparisons among the groups were analyzed by χ^2-test. $P < 0.05$ was considered indicative of statistical significance.

RESULTS

Association between nutritional status and severity of chronic liver disease

THE ALTERATION OF nutritional status assessed by BMI, AC, AMC, TSF, albumin, the PNI and CONUT according to the severity of chronic liver disease is demonstrated in Figure 1. Albumin level and PNI significantly decreased and the value of CONUT significantly increased according to the severity of chronic liver disease (Fig. 1e–g; $P < 0.001$). AC and AMC differed significantly among the groups (Fig. 1b,c; $P < 0.05$); however, we did not observe a decline in either AC or AMC according to severity of chronic liver disease. Similarly, TLC and TC differed significantly among the groups ($P < 0.001$) without a decline according to the severity of chronic liver disease (data not shown). In addition, no significant alterations were observed in BMI and TSF (Fig. 1a,d).

Association between anthropometry and albumin, PNI or CONUT

The alterations in BMI, AC, AMC and TSF according to changes in albumin, PNI or CONUT are demonstrated in Figure 2. All the values of BMI, AC, AMC and TSF significantly decreased as CONUT value increased (Fig. 2i–l; $P < 0.05$ for BMI and $P < 0.001$ for AC, AMC and TSF). AC and AMC significantly decreased with albumin level ($P < 0.01$ and 0.05, respectively), whereas BMI and TSF differed significantly among the groups ($P < 0.05$) without decreasing in accordance with changes in albumin (Fig. 2a–d). AC, AMC and TSF significantly decreased with PNI (Fig. 2f–h; $P < 0.01$ for AC and $P < 0.05$ for AMC and TSF). A decline in BMI was observed in accordance with a decrease in PNI, while BMI did not differ significantly among the groups (Fig. 2e).

Table 2 Assessment of malnutrition by controlling nutritional status

Parameter	Malnutrition degree			
	Normal	Mild	Moderate	Severe
Albumin (g/dL)	≥3.50	3.00–3.49	2.50–2.99	<2.50
Score	0	2	4	6
Total lymphocyte count (/mL)	≥1600	1200–1599	800–1199	<800
Score	0	1	2	3
Total cholesterol (mg/dL)	≥180	140–179	100–139	<100
Score	0	1	2	3
Total score	0–1	2–4	5–8	9–12

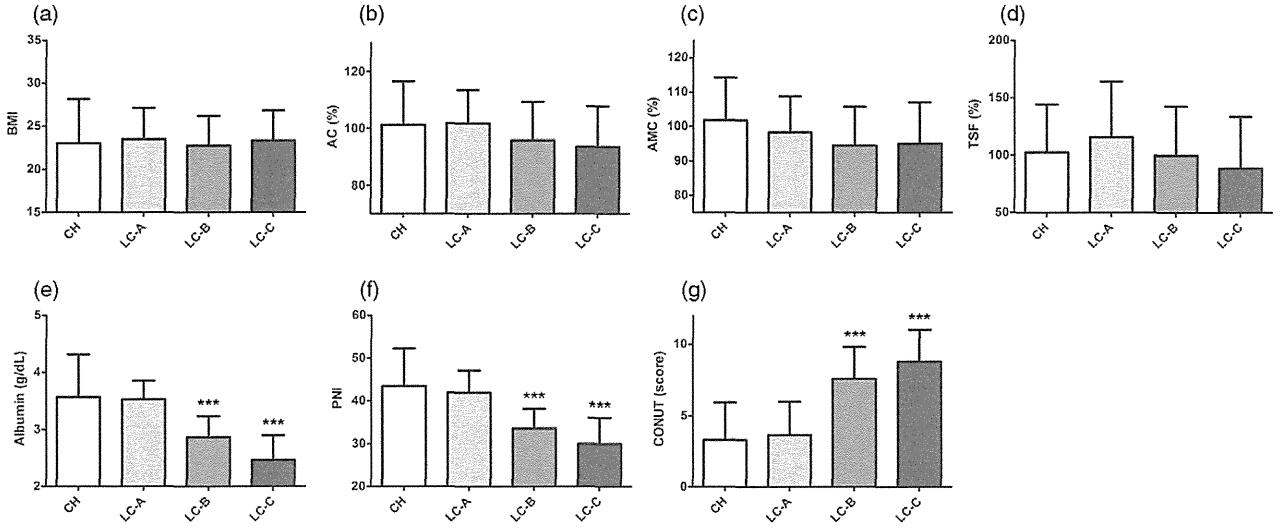

Figure 1 Alteration of nutritional status assessed by BMI (a), AC (b), AMC (c), TSF (d), albumin (e), PNI (f), or CONUT (g) according to severity of liver disease. AC, AMC, albumin, PNI and CONUT differed significantly among the groups by Kruskal–Wallis test ($P < 0.05$ for AC and AMC and $P < 0.001$ for albumin, PNI and CONUT). ***$P < 0.001$ in Dunn's multiple comparison tests to compare the pairs of groups (vs CH). AC, arm circumference; AMC, mid-arm muscle circumference; BMI, body mass index; CH, chronic hepatitis; CONUT, controlling nutritional status; LC, liver cirrhosis; PNI, prognostic nutritional index; TSF, triceps skinfold thickness.

Ratio of malnutritional status assessed by several methods

The alteration in the ratio of malnutritional status assessed by BMI, AC, AMC, TSF, SGA, the PNI and CONUT according to the severity of chronic liver disease is demonstrated in Figure 3. When BMI less than 18.5 was defined as malnutrition, nine had malnutrition among the 138 cirrhotic patients (6.5%). In addition, the ratio of malnutrition did not differ significantly among the groups (Fig. 3a). When AC%, AMC% or TSF% of less than 90% was defined as malnutrition, 45 (32.6%), 43 (31.2%) or 70 (50.7%) had malnutrition, respectively (Fig. 3b–d). In addition, the ratio of malnutrition assessed by TSF, but not AC or AMC, was significantly different among the groups ($P < 0.05$). When assessed by SGA, 75 (54.3%) had malnutrition and the ratio of malnutrition was significantly different among the groups ($P < 0.001$, Fig. 3e). On the other hand, 112 and 108 had moderate and severe malnutrition assessed by the PNI (81.2%) and CONUT (78.3%) and the ratio of malnutrition was significantly different among the groups ($P < 0.001$, Fig. 3f,g).

DISCUSSION

IN THIS STUDY, we demonstrated that the values of the PNI and CONUT were closely associated with both the severity of chronic liver disease and physical condition assessed by anthropometry. In addition, approximately 80% of cirrhotic patients were shown to have obvious malnutrition assessed by the PNI and CONUT.

In ordinary medical care, it should be important to establish simple assessments for nutritional status. However, the use of such standard methods for nutritional assessment remains unclear in patients with chronic liver disease.

Subjective global assessment is a standard and useful method for nutritional assessment based on simple questions and physical findings concerning bodyweight, appetite, pyrexia, vomiting, diarrhea, ascites, edema and wasting of muscle or fat.[18] However, our results demonstrated that SGA could underestimate nutritional status in patients with chronic liver disease except for advanced cirrhosis, which supports previous studies.[8,19] In addition, SGA fails to predict outcomes such as complications, transplantation and death in cirrhotic

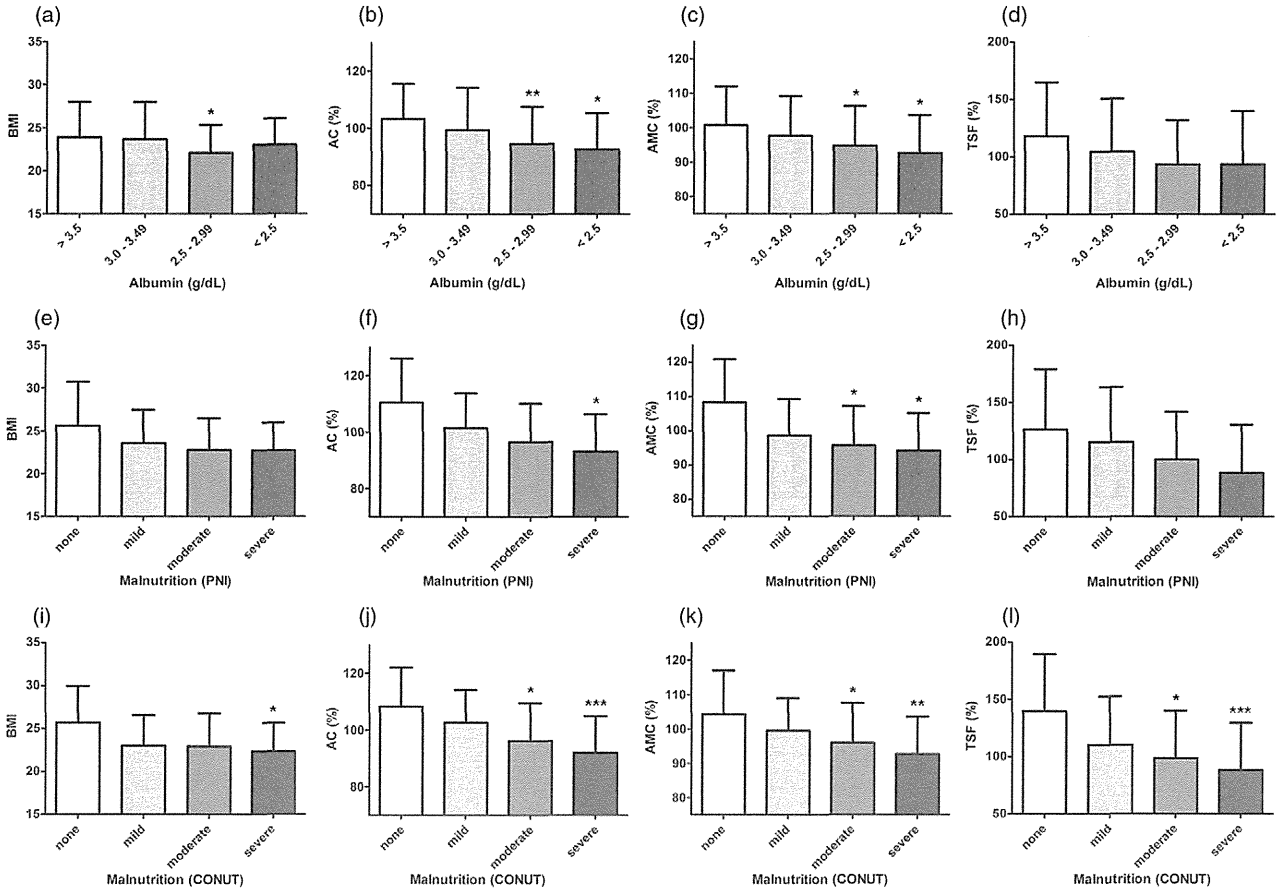

Figure 2 Alterations of BMI (a,e,i), AC (b,f,j), AMC (c,g,k) and TSF (d,h,l) according to changes in albumin level (a–d), PNI (e–h) or CONUT (i–l). BMI, AC, AMC and TSF in relation to albumin ($P < 0.01$ for AC and $P < 0.05$ for BMI, AMC and TSF), AC, AMC and TSF in relation to PNI ($P < 0.01$ for AC and $P < 0.05$ for AMC and TSF), and BMI, AC, AMC and TSF in relation to CONUT ($P < 0.05$ for BMI and $P < 0.001$ for AC, AMC and TSF) differed significantly among the groups by Kruskal–Wallis test. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ in Dunn's multiple comparison tests to compare the pairs of groups (vs albumin ≥3.5 g/dL or no malnutrition by PNI and CONUT). AC, arm circumference; AMC, mid-arm muscle circumference; BMI, body mass index; CONUT, controlling nutritional status; PNI, prognostic nutritional index; TSF, triceps skinfold thickness.

patients, except for postoperative outcome of liver transplantation.[28,29] Therefore, SGA is not sufficient for nutritional assessment of patients with chronic liver disease.

Anthropometry is another standard and useful method for nutritional assessment.[18] Nutritional assessment by anthropometry is beneficial because AMC or TSF is reported to be a predictive factor for survival, accompanied by an association with altered liver function in cirrhotic patients.[1,5] In this study, changes in TSF tended to be associated with alterations of liver function, when limited to cirrhotic patients ($P < 0.1$, data not shown). However, changes in AMC were not associated with alteration of liver function in cirrhotic patients (data not shown). Moreover, the association between alterations in TSF and liver function was disrupted when we considered all the patients with CH and LC. Although it is not clear why our results did not support previous studies, the difference in etiology between different countries for chronic liver disease might have been involved.[30–32] Regardless of the reason, nutritional assessment by anthropometry in patients with chronic liver disease is questionable for wider clinical application in terms of reproducibility. In addition, our results demonstrated that nutritional assessment by AC, AMC or TSF could also underestimate nutritional status in patients with chronic liver disease, similar to SGA.[2,8]

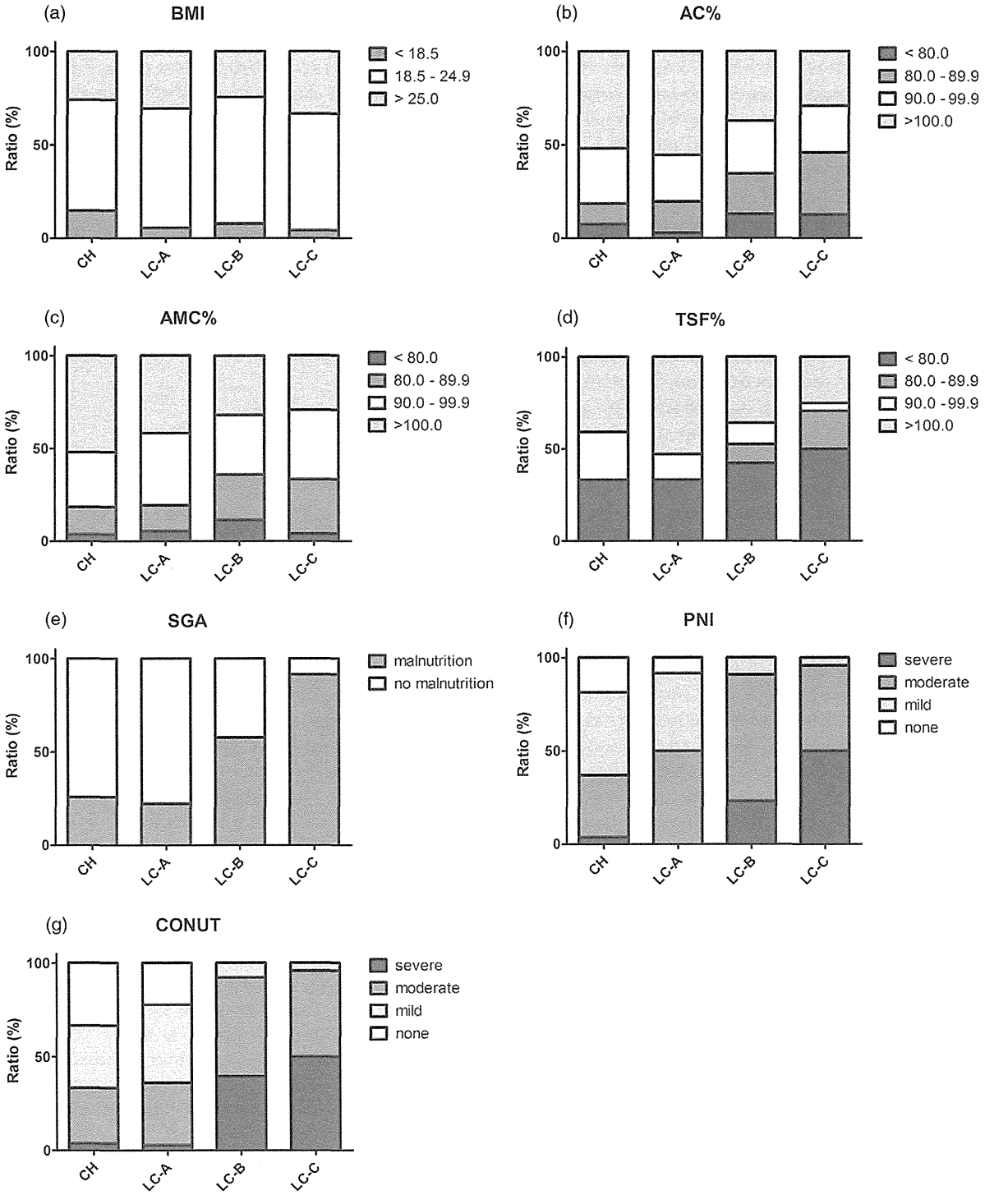

Figure 3 Ratio of patients with malnutritional status assessed by BMI (a), AC (b), AMC (c), TSF (d), SGA (e), PNI (f) and CONUT (g). The number of patients with malnutritional status significantly differed among the groups in TSF, SGA, PNI and CONUT analyzed by χ^2-test ($P < 0.05$ for TSF and $P < 0.001$ for SGA, PNI and CONUT). AC, arm circumference; AMC, mid-arm muscle circumference; BMI, body mass index; CH, chronic hepatitis; CONUT, controlling nutritional status; LC, liver cirrhosis; PNI, prognostic nutritional index; TSF, triceps skinfold thickness.

The PNI and CONUT are simple assessments for nutritional status that are constructed from only laboratory data.[23,25] Therefore, nutritional assessments by the PNI and CONUT could be candidates when considering widespread clinical application. The PNI has been demonstrated to predict the outcome in patients with gastrointestinal, pancreatic and gynecological cancer.[20–24] However, the usefulness of the PNI in patients with chronic liver disease remains unclear. In this study, we demonstrated that the PNI was associated with severity of chronic liver disease and AC, AMC or TSF. In addition, a decline in BMI was observed in association with a decrease in PNI. There are fewer data available for the usefulness of the clinical application of CONUT. In this study, CONUT showed a better association than PNI with anthropometric values, accompanied by an association with severity of chronic liver disease. CONUT was significantly associated with AC, AMC and TSF, as well as BMI. Taken together, these results suggest that CONUT is better than the PNI and could be a potential simple assessment of nutritional status in patients with chronic liver disease.

At present, simple bedside methods such as SGA or anthropometry are recommended to identify cirrhotic patients at risk of malnutrition.[18] Nutritional assessment by these methods might not be sufficient in patients with chronic liver disease because of underestimation of nutritional status, although these methods are known to be useful for nutritional assessment. In addition, assessment of SGA or anthropometry might differ among those performing the tests because each assessment requires technical expertise and experience. The PNI and CONUT are constructed from only two or three factors such as albumin, TLC and TC. Nutritional assessment by the PNI and CONUT is low cost and special techniques or experience is not required. Moreover, physical condition can be reflected by the PNI and CONUT because of the association between PNI or CONUT and anthropometric measurements such as BMI, AMC or TSF. In addition, it might not be difficult for the PNI and CONUT to be applied widely in a clinical setting because laboratory data such as albumin, TLC and TC are supposed to be measured ordinarily in patients with chronic liver disease.

Further studies about the usefulness of the PNI and CONUT are needed. We did not compare the PNI or CONUT and the other assessments for nutritional status such as RQ, or prediction of outcome such as complications or prognosis using the PNI or CONUT. However, approximately 80% of the cirrhotic patients suffered from obvious malnutrition, as assessed by the PNI or CONUT, which supports previous studies.[2,6,8,9] In addition, the PNI and CONUT values were closely associated with severity of chronic liver disease according to the Child–Pugh classification, which is one of the most predictive factors for prognosis in cirrhotic patients.[33–35] Therefore, the PNI or CONUT as a nutritional assessment tool has great potential in patients with chronic liver disease, especially for widespread clinical application.

In conclusion, the PNI and CONUT could have great potential for nutritional assessment in patients with chronic liver disease, and could be standard assessment tools in ordinary medical care, because of their simplicity.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Decreased expression of insulin and increased expression of pancreatic transcription factor PDX-1 in islets in patients with liver cirrhosis: a comparative investigation using human autopsy specimens

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Abstract
Background Glucose intolerance in patients with liver cirrhosis (LC), known as hepatogenous diabetes, is thought to be distinct from type 2 diabetes (T2DM) in some aspects. Hyperinsulinemia and/or insulin resistance in liver disease is associated with hepatocarcinogenesis, growth of hepatocellular carcinoma, and poor prognosis. However, the pathophysiological processes in islets that are responsible for hyperinsulinemia in LC are still not precisely known. Therefore, we investigated the histopathological differences in islets of Langerhans cells between LC and T2DM.
Methods A total of 35 human autopsy pancreatic tissue samples were used in this study (control, $n = 18$; T2DM, $n = 6$; LC, $n = 11$). The expression of insulin, glucagon, somatostatin, pancreatic duodenal homeobox-1 (PDX-1), proliferating cell nuclear antigen (PCNA), and Ki-67 was examined using immunohistochemistry and quantitated by image analysis.
Results Islet hypertrophy and a significant increase in PCNA-positive cells in islets were observed in the tissues from LC cases. The insulin-positive areas in islets were significantly decreased in LC cases compared with control and T2DM cases ($P = 0.001$, $P = 0.035$, respectively), whereas the PDX-1-positive area was significantly increased in LC cases ($P = 0.001$) compared with the control. Furthermore, disorganization of pancreatic endocrine cells and nucleocytoplasmic translocation of PDX-1 were both seen in the LC subjects.
Conclusions In LC, islets undergo hypertrophy and exhibit paradoxical expression of insulin and PDX-1. In the subjects autopsied, insulin expression was decreased, whereas expression of the pancreatic transcription factor PDX-1 was increased in LC. These results point to important distinctions between LC and T2DM.

Keywords Liver cirrhosis · Diabetes · Pancreatic duodenal homeobox-1 · Insulin · Islet

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Abbreviations

PDX-1	Pancreatic duodenal homeobox-1
PCNA	Proliferating cell nuclear antigen
BMI	Body mass index
HbA1c	Hemoglobin A1c

Introduction

Insulin, secreted by the pancreas, is one of the most important regulators of plasma glucose. Currently, it is thought that hyperglycemia in type 2 diabetes mellitus (T2DM) is caused by impaired insulin secretion and decreased β cell mass together with the development of insulin resistance [1]. On the other hand, glucose intolerance in patients with liver

cirrhosis (LC), known as hepatogenous diabetes, may differ from T2DM in important ways.

The liver plays a central role in regulating postabsorptive and postprandial glucose levels by means of glycogenolysis and gluconeogenesis [2–4]. Glucose is absorbed by the intestinal tract and reaches the liver via the portal vein. Approximately 60 % of glucose is converted to glycogen in the liver, and the remaining glucose is subsequently carried by the bloodstream to peripheral tissues such as adipose tissue and skeletal muscle, resulting in decreased postprandial glucose levels. On the other hand, in the postabsorptive state, the plasma glucose level is maintained by hepatic glycogenolysis and by release of glucose synthesized from amino acids or glycerol in the liver [4, 5]. In LC, postprandial hyperglycemia is observed, reflecting the depletion of hepatic parenchymal cells, which results in impaired glucose uptake and glycogen synthesis. The fasting plasma glucose level does not rise, reflecting impaired gluconeogenesis and glycogen depletion in LC [4, 6, 7]. Correspondingly, β cell function and insulin resistance are also important factors in glucose metabolism in LC [8]. Notably, hyperinsulinemia has been reported in hepatogenous diabetes [9]. However, the pathophysiological process responsible for hyperinsulinemia in LC is still not precisely known.

Hepatic parenchymal damage is thought to be an important factor in peripheral hyperinsulinism by decreasing the hepatic insulin degradation rate, and portosystemic shunts may promote postprandial hyperglycemia [10–12]. In fact, hyperinsulinemia is not always explained by compensatory hypersecretion of insulin from β cells, even in hepatitis C virus (HCV)-related liver diseases [10, 13]. However, Greco et al. [14] reported that hyperinsulinemia might be caused by increased pancreatic β cell sensitivity to glucose, even in patients with Child B grade LC. Furthermore, in HCV-related hepatitis in particular, without progression of LC, insulin resistance and hyperinsulinemia are frequently seen due to both direct and indirect involvement of the virus [13, 15–18]. Hyperinsulinemia, frequently accompanied by insulin resistance, is clearly associated with hepatocarcinogenesis, growth of hepatocellular carcinoma, and poor prognosis in liver disease [19–22]. The effect of antidiabetic therapy on hepatoma is still a matter of debate [23–25]. In order to design appropriate treatments for hepatogenous diabetes, etiologic and environmental factors in addition to pathological changes in β cells must be considered just as they are in the treatment of T2DM [26].

The mechanism of pathogenesis in T2DM has been widely discussed [1, 27, 28]. Recent work has focused on pancreatic duodenal homeobox-1 (PDX-1), a transcription factor that plays a pivotal role in differentiation and maintaining the function of pancreatic endocrine cells [29, 30]. In particular, PDX-1 is associated with compensatory β cell expansion and insulin secretory function [29]. Although hypertrophy of islets has been reported in LC and in HCV-core transgenic mice, no evidence exists of pathological changes in pancreatic endocrine cells in these conditions [16, 31–33].

The aim of this study was to investigate the morphological and endocrinological changes in islets of Langerhans in cirrhotic and diabetic patients, with a focus on PDX-1 expression in islet cells in both groups.

Materials and methods

Case selection and subjects

Human autopsy tissue samples were collected at Kurume University School of Medicine, Japan, and the study design was approved by the Institutional Review Board (IRB) of Kurume University School of Medicine. A total of 35 adult Japanese subjects who were autopsied in the Department of Diagnostic Pathology of Kurume University between the years 2000 and 2010 were retrospectively included, and tissue samples of the pancreatic tail were taken from each subject. Eighteen patients who had died of non-hepatic causes with no history of diabetes were selected for controls. Eleven patients with hepatitis C virus-related cirrhosis who died of hepatic diseases with no history of diabetic care were selected for the LC group. Six patients with T2DM who died of non-hepatic causes were selected for the T2DM group. Diabetes care in the T2DM group was as follows: no medication ($n = 3$) and sulfonylurea with metformin ($n = 3$). Subjects were excluded if they had exogenous insulin treatment or exhibited obesity [body mass index (BMI) more than 25], as determined on weighing after removal of the body cavity effusion at autopsy. The following clinical and laboratory data were assessed based on medical charts and preserved serum (Table 1). All of the blood samples were taken at fasting state in the morning during hospitalization: sex, age, BMI, levels of hemoglobin A1c (HbA1c) in the T2DM group, plasma glucose level, insulin level, and levels of C-peptide and free fatty acids in the LC group.

Immunohistochemistry (IHC)

Paraffin-embedded tissue samples were cut at 4 μm and mounted on coated glass slides and immunostained using one of the following automated systems: BenchMark XT (Ventana Automated Systems, Inc., Tucson, AZ, USA), DAKO autostainer (DakoCytomation, Glostrup, Denmark), or Bond-Max autostainer (Leica Microsystems, Newcastle, UK). Primary antibodies (with dilutions) were as follows:

Table 1 Characteristics, clinical and fasting laboratory data of subjects

	Reference value (fasting)	Control	LC	T2DM	*P*
Number		18	11	6	
Sex (F/M)		7/11	8/3	3/3	NS
Age (years)		65.2 ± 3.2	70.5 ± 1.4	67.7 ± 4.3	NS
BMI	22–25	20.2 ± 0.7	20.8 ± 0.7	19.2 ± 1.3	NS
HbA1c (%)	4.3–5.8			6.5 ± 0.5	N/A
Plasma glucose (mg/dL)	80–109		111.5 ± 15.0		N/A
Insulin (μIU/mL)	1.84–12.2		19.1 ± 4.2		N/A
C-peptide (ng/mL)	0.61–2.09		2.6 ± 0.6		N/A
Free fatty acid (μEq/L)	140–850		534.3 ± 117.4		N/A

Values are given as number or mean ± SD

BMI body mass index, *HbA1c* hemoglobin A1c, *NS* not significant, *N/A* not applicable

Ki67 (1:200, DakoCytomation, Glostrup, Denmark), insulin (1:400, Leica Microsystems, Newcastle, UK), glucagon (1:10,000, Leica Microsystems, Newcastle, UK), somatostatin (ready to use, Nichirei, Japan), PDX-1 (1:12,000, OriGene Technologies, MD, USA), and proliferating cell nuclear antigen (PCNA) (ready to use, Thermo Scientific, CA, USA).

Ki67 immunostaining was performed on the BenchMark XT system. Briefly, slides were heat-treated in CC1 retrieval solution (Ventana) for 60 min, and incubated with the Ki67 antibody for 30 min. The system used the streptavidin–biotin complex method with 3,3′-diaminobenzidine (DAB) as the chromogen (Ventana iVIEW DAB detection kit). Immunostaining for insulin, glucagon, and somatostatin was performed on a DAKO autostainer using the ChemMate ENVISION method (DakoCytomation, Glostrup, Denmark). Briefly, samples were incubated with primary antibody at room temperature for 30 min. After slides were washed in Tris-buffered saline (TBS), they were incubated with labeled polymer-HRP secondary antibody for 30 min at room temperature. After TBS washing, the staining was visualized using DAB for glucagon and somatostatin, andwith VIP (purple) for insulin (VIP substrate Kit, Vector Laboratories, Burlingame, CA, USA). Immunostaining with PDX-1 and PCNA was performed on the same Bond-Max system using onboard heat-induced antigen retrieval with ER2 for 10 min and a Refine polymer detection system (Leica Microsystems, Newcastle, UK). DAB was used as the chromogen.

We also used double staining to reveal insulin- and glucagon-positive areas in order to assess and compare expression areas accurately. Since somatostatin-positive cells are distinct from insulin/glucagon cells [34] and account for only 5–10 % of the islets, we used somatostatin staining alone to evaluate the somatostatin-positive area.

IHC expression area analysis

Ten fields in islet size order were selected from each tissue sample at 200×. Digital images were captured using a CCD digital camera (DS-Fil, Nikon, Tokyo). Expression analysis was performed by measuring the expression area using WinROOF software (version 5.7; Mitani Co., Osaka, Japan) [35, 36]. Expression areas were measured and averaged.

Statistical analysis

All data are expressed as mean ± SD. Comparisons among groups were performed by analysis of variance followed by post hoc tests (Fisher's PLSD).

Results

Characteristics of subjects

Clinical and fasting laboratory data of subjects are summarized in Table 1. There were no significant differences in sex, age, and BMI among the three groups.

Changes of islet area

The average area of each islet was significantly enlarged in the LC group and significantly reduced in the T2DM group compared to the control group (Figs. 1a, 2).

Changes of insulin/glucagon- and somatostatin-positive area

Insulin-positive area and glucagon-positive area in islets were significantly reduced in the LC group compared with the control group (Figs. 1c, d, 2). Insulin-positive area and glucagon-positive area of the T2DM group were also significantly decreased compared with the control group (Figs. 1c, d, 2). These changes were also observed by double staining of insulin and glucagon. Somatostatin-positive area was significantly increased in LC group and trend toward increasing in T2DM groups compared with the control group (Fig. 1e, 2). Moreover,

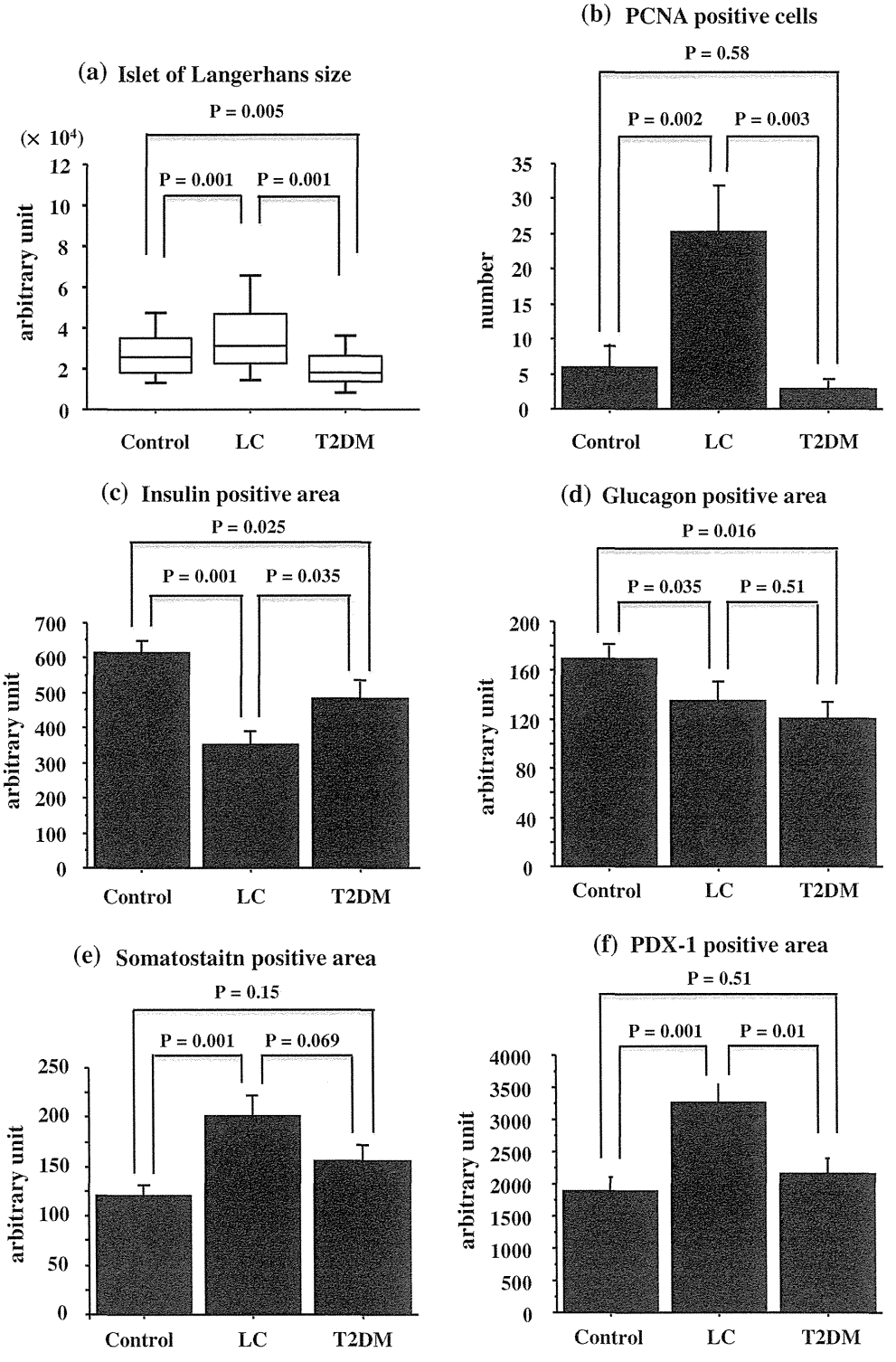

Fig. 1 Comparison of islet size and staining area. **a** Islet of Langerhans size, **b** number of PCNA-positive cells, **c** insulin-positive area, **d** glucagon-positive area, **e** somatostatin-positive area, **f** PDX-1-positive area

insulin-positive cells were located mainly in central regions of islets while glucagon-positive cells were located mainly at the periphery in control and DM groups; a disorganized distribution of these cells was apparent in the LC group (Fig. 2).

Number of PCNA-positive and Ki67-positive cells in islets

The number of PCNA-positive cells in islets was significantly increased in the LC group compared to the control

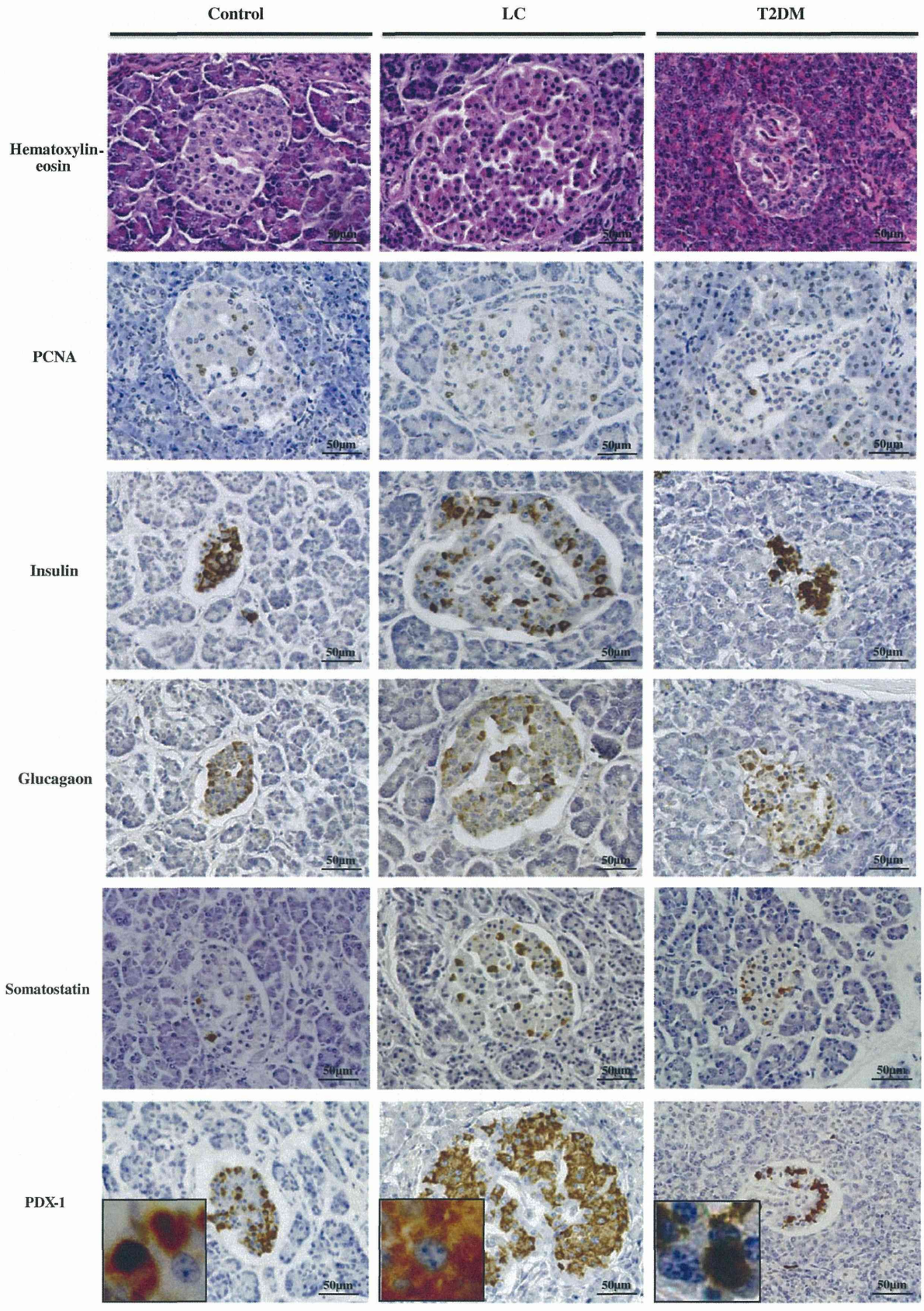

Fig. 2 Representative images of islets; magnification, ×200

group ($P = 0.002$, Fig. 1b). There was no significant difference in the number of PCNA-positive cells between the T2DM and control groups. Ki67-positive cells were detected in just one tissue sample of the LC group and were not observed in the other groups.

Expression of PDX-1

The PDX-1-positive area in islets was significantly increased in LC groups compared with the control (Fig. 1f). There was no significant difference in PDX-1 expression between the T2DM and control groups (Fig. 1f). In addition, nucleocytoplasmic translocation of PDX-1 was observed in the LC group, whereas PDX-1 was mainly expressed in the nuclei in the T2DM and control groups (Fig. 2, inset).

Discussion

Our findings demonstrate hypertrophy of islets in LC. However, insulin-positive area in islets was significantly decreased in the LC group compared with the control and T2DM groups. In contrast, the PDX-1-positive area was significantly increased in LC, and nucleocytoplasmic translocation of PDX-1 was seen in LC.

The area of islets was reduced in the T2DM group. Half of our cases had a history of diabetic therapy such as sulfonylurea. While compensatory islet hypertrophy is seen in early stages of diabetes, which results in maintenance of normal plasma glucose levels, collapse of the compensatory system and elevated β cell apoptosis results in reduced islet hypertrophy [37, 38]. Thus, the β cells might have been exhausted in the subjects we studied, which resulted in their need for antidiabetic therapy including sulfonylurea.

In contrast, we observed islet hypertrophy and an increase in PCNA-positive cells of islets in the LC group. These findings have a good correlation with previous reports [16, 31–33]. Compensatory insulin hypersecretion and expansion of β cell mass are caused by insulin resistance, obesity, and pregnancy [37, 39–42]. Notably, an interaction between the liver and pancreas in the regulation of glucose metabolism has been recently proposed. Loss of insulin signaling in hepatocytes leads to severe insulin resistance and islet hypertrophy [43–45]. In addition, pancreatic beta cell mass is regulated by neuronal signals from the liver [46]. Although we could not demonstrate insulin resistance in the LC group due to limited laboratory data, we excluded patients with obesity. The liver failure could also affect islet hypertrophy. Since we have no autopsy specimens in patients with HCV-related cirrhosis who died of non-hepatic diseases, there is a possibility that the liver failure rather than LC induced islet hypertrophy. However, Saitoh et al. [31] reported the islet hypertrophy in patients with LC and increases PCNA-positive cell counts in islet. Therefore, it seems that the LC rather than liver failure affected the changes in islets.

The discrepancy between PCNA and Ki-67 expressions in islets were seen in this study. Although Ki-67 is known to be a useful marker in cell proliferation with rapid turnover cells such as cancer, limited information is available for the usefulness in cells except cancer [47]. On the other hand, PCNA expressions have been previously used for evaluation of the islet cell cycle [31, 48]. Taken together, the discrepancy might be caused by the difference of characteristics between rapid and slowly growing cells.

Having examined the pathological changes in pancreatic endocrine cells, we now discuss several related issues.

Liver cirrhosis

Insulin and PDX-1

In this study, we observed islet hypertrophy and an increase in PCNA-positive cells of islets in the LC. Since islet size is generally defined by β cell mass [38, 49] and plasma insulin levels and C-peptide levels were maintained in our subjects, we expected insulin staining to delineate islets in LC. However, insulin-positive area in the islets of LC patients was decreased despite our observations of islet hypertrophy and disorganization of β cells. To characterize the islet cells which were negative for insulin, we focused on the PDX-1 which associated with compensatory β cell expansion and insulin secretory function. The result was that the PDX-1-positive area in islets of LC was increased. Therefore, these cells may have the characteristics of β cells. Since PDX-1 occurs in differentiated endocrine cells and is upregulated specifically in β cells, resulting in insulin secretary function [29, 50], increased PDX-1 expression may be an adaptive response to glucose intolerance in the LC. We also found that nucleocytoplasmic translocation of PDX-1 occurred in the LC groups. Kawamori et al. [51] demonstrated that nucleocytoplasmic translocation of PDX-1 indicates increased oxidative stress in islets. Therefore, it is possible that functional impairments, including defects in insulin synthesis, occur in LC despite compensatory hypertrophy of islets. We propose that there is unknown cross-talk between the liver and pancreas in LC.

Glucagon

In our study, glucagon-positive area in islets of LC patients was also reduced, accompanied by abnormal distribution. Although we could not measure the plasma glucagon levels

of our subjects, elevated plasma glucagon levels in LC have been reported [52, 53]. The reason for this discrepancy remains unclear. However, Antoniello et al. [54] reported no difference in glucagon degradation activity between healthy and cirrhotic subjects, using biopsy specimens. In addition, there are no reports of changes in α cells in LC. Further investigation should be focused on pathophysiology of hyperglucagonemia, in LC with and without a portosystemic shunt.

Diabetes

Decreased β cell mass and elevated plasma glucagon levels have been reported in advanced T2DM [37, 55]. In the T2DM group, we found that the area of islets was reduced and the insulin-positive area in islets were significantly decreased compared to the control group. Although we could not investigate serum glucagon levels, we also found that the glucagon-positive area in islets was decreased in T2DM. In addition, we examined the expression of PDX-1, an important transcription factor regulating islet cell function. Although overexpression of PDX-1 is reported in hyperinsulinemic obese mice, limited information is available regarding PDX-1 expression in β cells of diabetic patients [56]. We first demonstrated that PDX-1-positive area was maintained in T2DM patients including those who received antidiabetic medications. These findings indicate that PDX-1-positive cells, which have characteristics of β cells, are maintained even in medicated diabetic patients. Since α cells in islets are known to play an important role in insulin secretion [57], the insulin secretion capacity of β cells (which is one of their functions) might have been impaired in this study.

Somatostatin

Somatostatin, secreted by islet δ cells and the hypothalamus, is unique in its ability to suppress glucagon and insulin release from pancreatic endocrine cells [54, 55]. Exogenous somatostatin inhibits insulin and glucagon secretion, and somatostatin secretion from δ cells is stimulated by increased glucose [58]. However, the impact of δ cells on hepatogenous diabetes as well as T2DM is poorly understood. Here, we showed that the somatostatin-positive area in islets was significantly increased in LC groups. The increased somatostatin content of islets in diabetic rats has been reported [59, 60], and our data showed similar changes in islets of diabetic patients.

Conclusion

Here, we showed that in LC, the islets of Langerhans were enlarged, and insulin-positive area and glucagon-positive area in islets were decreased accompanied with abnormal distributions. The PDX-1-positive area in islets of cirrhotic patients was increased, accompanied by nucleocytoplasmic translocation of PDX-1. Thus, we have demonstrated impaired β cell function in LC. However, it remains unclear how long insulin secretory malfunction had been going on during the progress of liver diseases in the subjects of our study. Further studies focusing on the histopathological changes of pancreatic endocrine cells, associated with disease progression and age, might point to better diabetic treatments in liver diseases.

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

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ORIGINAL ARTICLE

Value of Highly Sensitive Fucosylated Fraction of Alpha-Fetoprotein for Prediction of Hepatocellular Carcinoma Recurrence After Curative Treatment

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Abstract
Background The fucosylated fraction of alpha-fetoprotein (AFP-L3) has been used as a diagnostic marker for hepatocellular carcinoma (HCC). Recently, a highly sensitive immunoassay using an on-chip electrokinetic reaction and separation by affinity electrophoresis (micro-total analysis system; μTAS) has been developed.
Aim The aim of this study was to investigate the relationship between changes in the serum AFP-L3 level measured by μTAS assay and recurrence of HCC after curative treatment.
Methods A total of 414 HCC patients who met the Milan criteria and underwent hepatectomy or radiofrequency ablation were investigated prospectively for the relationship between HCC recurrence and values of tumor markers.
Results There were significant differences in recurrence-free survival between groups with and without AFP-L3 elevation measured before and after treatment ($p = 0.024$ and $p = 0.001$ for before and after treatment, respectively). Multivariate analysis revealed that AFP-L3 status ($p = 0.002$) measured 1 month after treatment was a significant independent predictor of HCC recurrence after curative treatment.
Conclusions Elevation of the serum AFP-L3 level before treatment is a predictor of HCC recurrence, and sustained elevation of the AFP-L3 level after treatment is an indicator of HCC recurrence. Repeated measurement of μTAS AFP-L3 should be performed for surveillance of HCC recurrence after curative treatment.

Keywords Hepatocellular carcinoma · Alpha-fetoprotein · Fucosylated fraction of alpha-fetoprotein · Des-gamma-carboxy prothrombin

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignancy and the third most common cause of cancer-related death [1]. Three serum markers, alpha-fetoprotein (AFP), fucosylated fraction of AFP (AFP-L3), and des-gamma-carboxy prothrombin (DCP), have been widely used for HCC surveillance and diagnosis. Among these markers, AFP is the most frequently used as a diagnostic marker for HCC in Japan [2, 3]. However, AFP levels are sometimes elevated in patients with chronic hepatitis and cirrhosis who have no evidence of HCC [4–6]. In contrast, AFP-L3 has been accepted as a specific marker for HCC by several investigators [7–9]. Moreover, its level predicts the malignant potential of HCC, and thus the expected outcome after treatment [10–15]. However, measurement of AFP-L3 has not always been reliable using serum samples with a low total AFP concentration determined by conventional lectin affinity electrophoresis, or using a liquid-phase binding assay system (LiBASys assay) [16]. Recently, a highly sensitive immunoassay for AFP-L3 using the on-chip electrokinetic reaction and separation by affinity electrophoresis (micro-total analysis system; μTAS) has been developed [17, 18]. We have already reported that the μTAS AFP-L3 is more sensitive for discriminating HCC from benign liver disease than the conventional LiBASys AFP-L3, particularly in subgroups with lower AFP concentrations and early stage HCC [19]. However, there has been little information about the μTAS AFP, AFP-L3 and DCP measured after curative treatment of HCC.

In the present study, we investigated the relationship between the tumor markers μTAS AFP, AFP-L3, and DCP, measured before and after curative treatment, and the likelihood of HCC recurrence. Additionally, we examined the changes in these markers in relation to treatment outcome.

Methods

Patients

A total of 420 HCC patients who met the Milan criteria (single tumor ≤5 cm in size or ≤3 tumors each ≤3 cm in size, and no macrovascular invasion) and who underwent hepatectomy or radiofrequency ablation at ten participating hospitals (Niigata University Medical and Dental Hospital, Medical Hospital of Tokyo Medical and Dental University, Kurume University Hospital, Tokyo Medical University Hospital, Juntendo University Hospital, Musashino Red Cross Hospital, Nihon University Itabashi Hospital, The University of Tokyo Hospital, Tokyo Women's Medical University Hospital, Hyogo Medical College Hospital), between May 2008 and November 2009, were investigated prospectively. HCC recurrence was assessed using several imaging modalities every 3 months after treatment, and all recurrences were evaluated up to the end of January 2012. The mean observation time after treatment was 594 ± 319 days. Six patients were excluded from the study because their follow-up periods were less than 1 month, giving a final total of 414 patients who were enrolled. Among these patients, HCC recurrence was observed in 236 patients, and the remaining 178 showed no HCC recurrence within the study period. Informed consent was obtained from each patient, and the study protocol conformed with the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in the a priori approval granted by each institution's human research committee.

Diagnosis of HCC and Laboratory Examination

Hepatocellular carcinoma patients were diagnosed using several imaging modalities, including computed tomography (CT), magnetic resonance imaging (MRI), and CT, during hepatic arteriography, giving due consideration to hyperattenuation in the arterial phase with washout in the late phase. Vascular invasion was evaluated on the basis of imaging modalities. In some cases that showed atypical features on imaging, ultrasound-guided biopsies were performed.

Simultaneous quantitative measurement of AFP-L1 (ng/ml) and AFP-L3 (ng/ml) was performed using the μTAS assay (Wako Pure Chemical Industries Ltd., Osaka, Japan) [17, 18]. Total AEP concentration (ng/ml) in the serum sample was determined by summation of AFP-L3 and AFP-L1, and then the percentage of the AFP-L3 level was calculated. The serum level of des-gamma-carboxy prothrombin (DCP) was also measured using a μTAS assay, except for patients who had taken warfarin, a DCP-inducing agent. The lower limits of quantitation for AFP (L1 and L3) and DCP were 0.3 ng/ml and 5 mAU/ml, respectively [17, 18]. When AFP-L3 was not detectable, the percentage of AFP-L3 was defined as 0.5 %. AFP, AFP-L3, and DCP were measured in the same serum sample before and 1 month after treatment. Cut-off positivity values for AFP, AFP-L3, and DCP were set at 20 ng/ml, 10 %, and 40 mAU/ml, respectively. Hepatic functional reserve was ranked using the criteria of the Child-Pugh scoring system.

Treatment of HCC

Therapeutic modalities for HCC patients were chosen on the basis of hepatic functional reserve, tumor multiplicity, and tumor size. Among the 414 HCC patients, 228 underwent hepatic resection and 186 underwent