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Body surface area is an independent factor contributing to the effects of lamivudine treatment

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Abstract

Background: It has been suggested that lamivudine therapy may be even more effective if administered at higher doses than is dictated by the current standard regimen. We analyzed the correlation between the effects of lamivudine and body surface area (BSA).
Method: We evaluated 134 patients with chronic hepatitis B who had been treated with lamivudine for more than 12 months. The effect of the treatment was evaluated from the levels of serum alanine aminotransferase (ALT) and HBV–DNA. Several variables that could influence the response to treatment, including ALT, albumin, and bilirubin levels, platelet counts, BSA, HBV–DNA, and HBeAg were analyzed.
Results: Univariate logistic analysis selected platelet counts, BSA, HBV–DNA and HBeAg in the biological evaluation, and bilirubin, BSA, HBV–DNA and HBeAg in the virological evaluation ($\chi^2 > 1.0$). Using these factors, multivariate analysis revealed that BSA ($\chi^2 = 12.8$, $p = 0.0004$) was the only factor that could contribute significantly to the improvement of ALT levels, and that BSA ($\chi^2 = 4.4$, $p = 0.0354$) and HBeAg ($\chi^2 = 8.1$, $p = 0.0044$) were independent factors that could influence the suppression of HBV–DNA.

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Conclusion: We revealed that BSA is a significantly predictor of the effect of lamivudine therapy, suggesting that lamivudine dosage should be based on the individual BSA.

Keywords: Lamivudine; Hepatitis B virus (HBV); Body surface area (BSA); Dose

1. Introduction

Chronic hepatitis B is an important cause of morbidity and mortality resulting from cirrhosis-related liver failure and hepatocelluar carcinoma (HCC) [1,2]. Lamivudine is a nucleoside analogue with activity against hepatitis B virus (HBV) replication. A daily dosage of lamivudine of 100 mg has been accepted worldwide for the treatment for chronic hepatitis B, since early studies showed that there was no significant difference in the effect of lamivudine at doses of 100 mg and 300 mg [3,4]. However, to establish ideal dosages in those studies, efficacy was mainly evaluated by measuring HBV–DNA, and the assay used was not as sensitive as the PCR assay [5,6]. Studies in which HBV–DNA was measured by PCR assay reported an additional viral suppressive activity with high doses (300 mg) of lamivudine for 24 weeks [7]. In addition to limits imposed by the assay methods used, the observation periods in the studies on lamivudine doses of 100 mg to 300 mg were limited to a period of 12 [3] or 24 weeks [4], because 1 year treatment with lamivudine often resulted in the relapse of HBV viremia [8]. Furthermore, the major drawback of lamivudine monotherapy is the emergence of resistant HBV with mutations of the tyrosine–methionine–aspartate–aspartate (YMDD) motif. The incidence of these mutants rises from 15 to 20% in the first year of therapy to 40% by the second year, and to 67% by the fourth year [9]. Although the effect of doses greater than 100 mg on the emergence of YMDD mutants has not been evaluated, base line body mass index has been reported to be significantly related to the emergence of mutation of HBV during lamivudine treatment in patients with co-infection of HBV and human immunodeficiency virus-1 HIV-1 [10]. Therefore, we evaluated the relationship between lamivudine effects and body surface are (BSA).

2. Patients and methods

2.1. Patients

A total of 134 patients with chronic hepatitis B were evaluated. Patients with fatty liver, patients with viral hepatitis C, patients with alcoholic abuser and patients with autoimmune disorder such as autoimmune hepatitis and primary biliary cirrhosis were excluded. They had been treated with 100 mg of lamivudine for more than 12 months at Kyushu University Hospital and its related hospitals (Table 1). For all patients, the existence of serum HBV–DNA was confirmed by TMA assay ($10^{3.7}$–$10^{8.7}$ genome equivalents/mL; 3.7–8.7 log genome equivalents [LGE]/mL) (Chugai Diagnostic Science, Tokyo, Japan) or by a Roche Monitor kit ($10^{2.6}$–$10^{7.6}$ copies/ml; 2.6–7.6 log copies/mL) (Roche Diagnostics, Tokyo, Japan) before treatment. None of the patients dropped out and all were treated with 100 mg/day lamivudine until the end of the observation period. After the start of medication, basic hepatic function and serum levels of HBV–DNA were measured at least every 3 months for all patients. The efficacy of lamivudine was evaluated from the serum levels of alanine aminotransferase (ALT) and HBV–DNA, as biological and virological effects, respectively. The evaluation using serum ALT was done as follows: (1) sustained responder- (SR-) ALT: the serum levels of ALT decreased, and remained at less than 30 U/L continuously during the observation; (2) transient responder- (TR-) ALT: the serum ALT decreased to less than 30 U/L, but increased to more than 30 U/L again during the subsequent observation; (3) non-responder- (NR-) ALT: the serum ALT always remained at more than 30 U/L throughout the observation. Similarly, the virological evaluation by HBV–DNA was done as follows, using a Roche Monitor kit: (1) SR-HBV: serum HBV–DNA decreased to levels undetectable by PCR (<2.6 log copies/mL), and remained negative continuously during the observation; (2) TR-HBV: the serum HBV–DNA decreased to undetectable levels once (<2.6 log copies/mL), but become positive again during the subsequent observation; (3) NR-HBV: the serum HBV–DNA was never negative throughout the observation (>2.6 log copies/mL). BSA was calculated using the method of DuBois.

Table 1
Characteristics of the 134 patients at base line[a]

	Total
Age	48.9 ± 11.4 (21–70)
Male/Female	98/36
CH/LC (Child A/B/C)	83/51 (35/9/7)
Observation period (month)	23.1 ± 7.9 (12–40)
ALT (U/L)	184.6 ± 243.4 (17–1722)
Albumin (g/dl)	3.76 ± 0.57 (2.3–4.9)
Bilirubin (mg/dl)	1.41 ± 1.79 (0.4–12.9)
Platelet ($10^4/\mu l$)	13.62 ± 6.19 (2.6–29.9)
BSA (m^2)[a]	1.702 ± 0.189 (1.28–2.24)
Height (m)	1.644 ± 0.084 (1.44–1.85)
Weight (kg)	64.2 ± 11.7 (38.0–105.0)
<5.0 (LEG/ml)	24
5.0 < ≤ 6.0	15
6.0 < ≤ 7.0	35
>7.0	48
Positive	77
Negative	57

[a] Plus-minus values are means ± S.D.

2.2. Statistical analysis

The backgrounds of the patients at the beginning of lamivudine treatment are shown as mean ± S.D. for the quantitative variables. Differences in the backgrounds of the SR, TR and NR-patients were examined by one-way ANOVA or χ^2-test. In order to confirm the contribution of the variables toward the effect of the treatment, univariate and multivariate logistic analysis were performed. For multivariate logistic analysis, we analyzed BSA as an independent factor contributing to effects of lamivudine treatment variables that showed χ^2 values of more than 1.0 in the univariate logistic model.

3. Results

Differences in background at the beginning of lamivudine treatment among the variables were evaluated by one-way ANOVA or χ^2-test. In the studies evaluating ALT (the biological response), the mean values of BSA and body weight were significantly lower in the SR-ALT group than in the other groups, and there was no significant difference in sex, observation period, height, ALT, albumin, bilirubin, platelet counts, HBV–DNA, or HBeAg among the groups (Table 2). Fig. 1 shows the distribution of BSA in each group, and demonstrated that there was an inverse relationship between BSA and the effects of lamivudine ($p=0.0394$ at SR versus TR and $p=0.0004$ at SR versus NR by Fisher's PLSD test). Similarly, the evaluation of HBV–DNA (the virological response) showed that BSA and body weight were also significantly lower in the SR-HBV group than in NR-HBV group (Table 3 and Fig. 1 [BSA: $p=0.0087$ at SR versus NR by Fisher's PLSD test]). The prevalence of HBeAg differed significantly among the groups ($p<0.005$ by χ^2-test), and in the NR-DNA group, most of the cases were positive for HBeAg (Table 3). The observation period also differed significantly among the groups ($p<0.05$), and the SR-DNA group had longer observation period than the others. There was no difference in sex, height, ALT, alubumin, bilirubin, platelet counts, or HBV–DNA among the groups.

Table 2
Evaluation using the serum levels of ALT

	SR	TR	NR
Number	60	43	31
Male/Female	39/21	31/12	28/3
Observation period (month)	22.3 ± 8.8	23.9 ± 7.1	23.5 ± 7.3
ALT (U/L)	196.7 ± 195.2	181.2 ± 263.9	165.6 ± 299.6
Albumin (g/dl)	3.82 ± 0.51	3.66 ± 0.67	3.80 ± 0.54
Bilirubin (mg/dl)	1.42 ± 2.18	1.58 ± 1.75	1.16 ± 0.70
Platelet ($\times 10^4\ \mu l^{-1}$)	14.49 ± 6.25	13.36 ± 6.31	12.30 ± 5.83
BSA (m^2)*	1.644 ± 0.184	1.719 ± 0.184	1.790 ± 0.172
Height (m)	1.629 ± 0.087	1.650 ± 0.091	1.667 ± 0.060
Weight (kg)*	60.45 ± 10.41	65.24 ± 10.53	70.10 ± 13.27
HBV–DNA			
<5.0 (LEG/ml)	11	8	5
5.0 < ≤ 6.0	6	1	8
6.0 < ≤ 7.0	17	11	7
>7.0	26	23	11
HBeAg			
Positive	32	25	20
Negative	28	18	11

* $p<0.005$.

In order to confirm the contribution of the variables toward the treatment, univariate and multivariate logistic analysis were performed. In univariate logistic analysis, platelet counts, BSA, body weight, height, HBV–DNA and HBeAg in the biological evaluation, and bilirubin, BSA, body weight, height, HBV–DNA and HBeAg in the virological evaluation, had χ^2 values of more than 1.0 (Table 4). Therefore, we used these factors except body weight and height as variables for multivariate logistic analysis, because there was a strong correlation between BSA and body weight ($R^2=0.89072$, $p<0.0001$), or BSA and height ($R^2=0.67708$, $p<0.0001$). The results of multivariate analysis revealed that BSA was the only significant factor that could contribute to the improvement of ALT levels ($\chi^2=12.8$, $p=0.0004$), and BSA and HBeAg were independent factors that could influence the disappearance of serum HBV–DNA (BSA: $\chi^2=4.4$, $p=0.0354$ and HBeAg: $\chi^2=8.1$, $p=0.0044$) (Table 5).

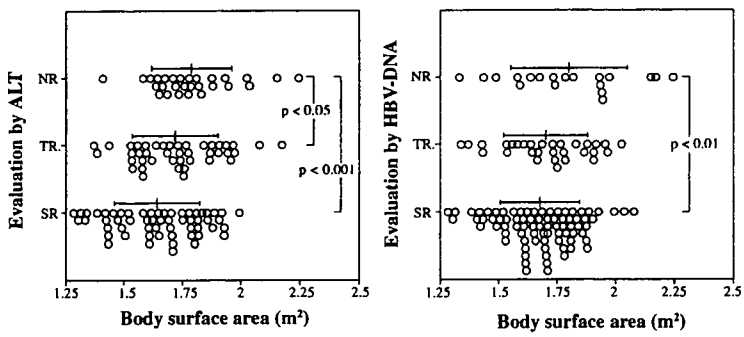

Fig. 1. The distribution of BSA according to the effect evaluated by ALT (left panel) and by HBV–DNA (right panel) was shown (open circle). The bars represent mean ± S.D. Non responders had significantly larger BSA than sustained responders in both evaluations.

Table 3
Evaluation using the serum levels of HBV–DNA

	SR	TR	NR
Number	83	31	20
Sex	60/23	22/9	16/4
Observation period (month)*	22.6 ± 7.5	26.7 ± 7.7	19.6 ± 7.7
ALT (U/L)	193.7 ± 246.6	188.5 ± 296.3	138.2 ± 91.4
Albumin (g/dl)	3.74 ± 0.58	3.78 ± 0.52	3.83 ± 0.61
Bilirubin (mg/dl)	1.56 ± 2.16	1.30 ± 0.98	0.92 ± 0.43
Platelet ($\times 10^4\ \mu l^{-1}$)	13.51 ± 6.05	13.04 ± 6.32	15.04 ± 6.70
BSA (m^2)*	1.676 ± 0.170	1.702 ± 0.181	1.801 ± 0.248
Height (m)	1.629 ± 0.087	1.650 ± 0.091	1.667 ± 0.060
Weight (kg)*	62.40 ± 10.03	64.33 ± 10.14	71.59 ± 17.26
HBV–DNA			
<5.0 (LEG/ml)	18	6	0
5.0 < ≤ 6.0	10	4	1
6.0 < ≤ 7.0	20	8	7
>7.0	35	13	12
HBeAg**			
Positive	40	19	18
Negative	43	12	2

* $p < 0.05$.
** $p < 0.005$.

Table 4
Univariate analysis on the lamivudine effects

Variable	χ^2	p-value
ALT evaluation		
ALT	0.35543766	0.5511
Albumin	0.31157046	0.5767
Bilirubin	0.19658636	0.6575
Platelet	2.68462623	0.1013
BSA	13.060725	0.0003
Height	4.09176033	0.0431
Weight	12.734481	0.0004
HBV–DNA	3.05562744	0.3831
HBeAg	1.02785409	0.3107
HBV–DNA evaluation		
ALT	0.4754853	0.4905
Albumin	0.39014806	0.5322
Bilirubin	2.42720044	0.1192
Platelet	0.25597276	0.6129
BSA	5.54675909	0.0185
Height	1.24367306	0.2648
Weight	5.45864661	0.0380
HBV–DNA	4.18232253	0.2424
HBeAg	10.2577192	0.0014

4. Discussion

In this present study, we found that BSA was a significant factor that could contribute to both the improvement of ALT levels (biological response) and the disappearance of serum HBV–DNA (virological response). Because the pharmacokinetics of lamivudine correlate with body weight, as is the case with many other drugs [11], it is reasonable to conclude that patients with lower BSA would have the higher blood concentration of lamivudine, although we did not actually monitor the lamivudine blood concentration. Recent reports suggest

Table 5
Multivariate analysis of the effects of lamivudine using potential univariate predictors

Variable	χ^2	p-value
ALT evaluation		
Platelet	3.2287548	0.0724
BSA	12.7808491	0.0004
HBV–DNA	2.42555739	0.4889
HBeAg	0.93377434	0.3339
HBV–DNA evaluation		
Bilirubin	3.76147767	0.0524
BSA	4.42516561	0.0354
HBV–DNA	2.23355431	0.5254
HBeAg	8.10893404	0.0044

that baseline body mass index is significantly related to the emergence of HBV mutation during lamivudine treatment (300 mg/day, >6 months) in patients coinfected with HBV and HIV-1 [10]. Therefore, the results of our study question whether a lamivudine dosage of 100 mg/day is adequate, particularly for long-term treatment.

The standard lamivudine dose of 100 mg daily was based on early studies in which doses of 25, 100, 300 mg were compared for 12 [3] or 24 weeks [4]. Dienstag et al. [3] reported that levels of HBV–DNA became undetectable in 70% of the patients who received the 25 mg dose of lamivudine ($n = 10$) and 100% of those treated with doses of 100 mg ($n = 11$) or 300 mg ($n = 11$). Nevens et al. [4] reported that HBV–DNA was undetectable at the end of 24 weeks of lamivudine treatment in 58% (25 mg), 93% (100 mg), and 88% (300 mg) of patients ($n = 16$, 19 and 19, respectively), and that abnormal ALT levels at baseline were normalized in 64% (25 mg), 45% (100 mg), and 36% (300 mg) of the patients at treatment completion. Because there were no significant differences reported in the rates of non-detection of HBV–DNA and nomalization of ALT levels between the 100 and 300 mg dose, the dose of 100 mg has become accepted as a therapeutic standard. However, several issues should be considered when evaluating the results of these studies, including: (1) number of patients; (2) period of treatment; (3) emergence of lamivudine-resistant mutants in long-term treatment; and (4) detection limit of HBV–DNA.

As we showed in the present study of 134 cases, there was a significant difference in the effect of BSA on both the biological and virological response at the end of observation period (mean = 2 years), although the differences in the mean values of BSA in each group were relatively small. Therefore, it is possible that previous studies failed to detect a significant contribution of BSA to the effects of lamivudine because of the smaller numbers of patients examined.

Observation periods of 24 weeks are not adequate for detecting the emergence of lamivudine-resistant mutants, since the incidence of the mutants rises from 15 to 20% in the first year to 40% by the second year and to 67% by the fourth year of treatment [9]. In our present study of HBV–DNA evaluation, the rate of TR-DNA and NR-DNA was 38% in patients

who were positive for HBV-DNA (>2.6 log copies/mL) at the end of observation (average of about 2 years). We did not confirm the YMDD mutation in all cases of TR-DNA and NR-DNA, and further study will be needed to confirm whether BSA affects the incidence of YMDD mutants.

It is important to consider the sensitivity of measurement of HBV-DNA in evaluating the effects of lamivudine, because a decrease in HBV-DNA is reported to be associated with a lower incidence of YMDD mutants [12]. In previous studies, which showed no difference in the effects of 100 and 300 mg lamivudine on HBV-DNA reduction (as described above) [3,4], HBV-DNA was measured quantitatively by liquid hybridization assay (Abbott Laboratories), which has a detection limit of 10^7 geq/mL [13]. Honkoop et al. studied the efficacy of 100 and 300 mg lamivudine in viral suppression for 24 weeks using a semi-quantitative PCR method with a detection limit of 10^2–10^3 geq/mL [7]. They showed that 29 and 37% patients were HBV-DNA negative after 24 week treatment with 100 and 300 mg lamivudine, respectively, indicating the possibility of additional viral suppressive activity with higher doses and longer-term therapy with lamivudine. Furthermore, using a more sensitive real-time quantitative PCR method with a detection limit of 1.7 log copies/mL, Ide et al. [12] reported that neither emergence of YMDD mutants nor a virological breakthrough of serum HBV DNA was observed in patients with <1.7 copies/mL. In our study, we measured HBV-DNA with a Roche Monitor kit, which has detection limit of 2.6 log copies/mL; 23% patients (TR-DNA) were HBV-DNA negative once, but became positive again during the subsequent observation. The TR-DNA group had a longer observation period than the other groups, this difference might affect the result that there was no significant difference in BSA between TR-DNA and SR-DNA groups, or TR-DNA and NR-DNA groups in the evaluation of HBV-DNA (Fig. 1). More precise analysis with longer observation period and/or using the real-time PCR method might show a clearer relationship between BSA and virological response with lamivudine.

In conclusion, we have revealed that BSA is a statistically significant and potentially important factor that can predict the effect of lamivudine therapy for chronic hepatitis B. A noteworthy finding in our study was that small differences in BSA could significantly influence the effect of the lamivudine treatment, suggesting that a small dose increase might increase the efficacy of lamivudine therapy. We believe that a long-term clinical trial with higher dose lamivudine treatment and a large number of cases is warranted, since lamivudine will continue to be a first-line treatment for HBV.

Appendix A

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• *CLINICAL RESEARCH* •

High relative fat-free mass is important for maintaining serum albumin levels in patients with compensated liver cirrhosis

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Abstract

AIM: In patients with liver cirrhosis, hypoalbuminemia causes edema and ascites, and a reduction in the quality of life. Since musculature is catabolized to supply amino acids for albumin synthesis in malnutritional cirrhotic patients, muscular volume is hypothesized to play an important role in albumin production. Therefore, we investigated the correlation between serum albumin levels and the fat-free mass (FFM) in cirrhotic patients.

METHODS: Fifty-seven patients (26 males and 31 females) with compensated liver cirrhosis were evaluated. Patients with edema or ascites were excluded from the study. Healthy volunteers (n = 104; 48 males and 56 females) were also evaluated as controls. FFM was measured using 5-500 kHz multifrequency bioelectric impedance analysis. To minimize the difference in FFM distribution between males and females, we introduced a new marker, relative FFM (rFFM), which represents the ratio of FFM in a patient relative to that in a volunteer of the same height. Following FFM measurement, the serum albumin levels of patients were assayed monthly.

RESULTS: In patients with active cirrhosis (alanine aminotransaminase [ALT] >50 U/L), both albumin (the difference between maximum and minimum levels) and the standard deviation of albumin levels (SD-albumin) during the observation period showed a significant correlation with rFFM. Multiple linear regression analysis using variables such as rFFM, platelet number, and serum cholesterol levels, choline esterase, albumin, bilirubin, and ALT revealed that rFFM and ALT were significant and independent factors that influenced albumin or SD-albumin in cirrhotic patients.

CONCLUSION: Our results indicate that cirrhotic patients with high rFFM showed less of a decrease in albumin levels, and that the muscle volume is one of the most important factors for maintaining serum albumins level in active cirrhosis. Exercise and protein-rich nutrition at the early stage of liver cirrhosis may be advisable for maintaining or increasing muscular volume.

Key words: Muscular volume; Fat-free mass (FFM); Multifrequency bioelectric impedance analysis; Albumin; Liver cirrhosis

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INTRODUCTION

In earlier reports, several factors were established for predicting the prognosis of patients with liver cirrhosis, such as ascites, liver volume, encephalopathy, esophageal varices, serum albumin, serum bilirubin and clotting factors[1-5]. Among these factors, serum albumin level was commonly regarded as the most reliable. The usefulness of utilizing hepatic protein synthesis as a parameter for predicting the prognosis of cirrhotic patients is supported by a series of reports, which showed that a medication with branched amino acid elevated the serum levels of albumin and improved the prognosis[6-8]. Besides predicting survival, maintaining high albumin levels is clinically important for patients with liver cirrhosis, because decreased serum albumin levels cause ascites and edema, which lower the quality of life.

With respect to protein metabolism in cirrhotic patients, it should be emphasized that musculature plays an important role as an amino-acid pool. In healthy controls, the amino acid pool is distributed in the musculature (80%), liver (15%) and plasma (5%)[9]. In cirrhotic patients, starvation readily induces muscle protein catabolism because there is relatively little glycogen stored in the cirrhotic liver. Therefore, in the assessment of nutritional status in cirrhotic patients, the evaluation of muscle protein stores is important.

Bedrest is commonly prescribed for the management of patients with liver cirrhosis. However, it is obvious that a bedrest results in muscle volume reduction, which might reduce the serum levels of albumin. Therefore, it seems reasonable to postulate that cirrhotic patients with high muscle volume would be more resistant to reductions in

serum albumin associated with the progression of liver damage. In order to confirm this hypothesis, we examined the relationship between serum albumin levels and body composition in cirrhotic patients.

A number of techniques are available for measuring body compartments, such as dual energy X-ray absorptiometry (DEXA), total body potassium measured by whole-body potassium-40 counting, total body water measured by isotope dilution, *in vivo* neutron activation analysis (IVNAA), and bioelectric impedance analysis (BIA). Those techniques, except BIA, associated with high cost and labor-intensive methods. Although BIA has been used to estimate fat-free mass (FFM) of cirrhotic patients at bedside, the validity of the measurements obtained with the single-frequency method has been questioned. Borghi (1996) showed that multifrequency BIA might yield valid body composition data for cirrhotic patients without ascites[10]. In the present study, we used multifrequency BIA for measuring FFM and we limited the study population to cirrhotic patients without ascites or edema.

MATERIALS AND METHODS

Patients

Fifty-seven patients with compensated liver cirrhosis, who were outpatients of our department from April 2000 to March 2002, were evaluated (Table 1). They consisted of 26 males and 31 females, and ranged in age from 27 to 83 years. Fifty-two (92%) and five (8%) patients were classified as Child A and Child B without edema and ascites respectively. Fifty (88%) and 7 (12%) cases were caused by hepatitis C virus and hepatitis B virus, respectively. There was no significant difference in the basic characteristics between males and females (Table 1). Patients with edema were excluded from this study. Ultrasonography (US) was done to confirm that the evaluated patients did not have ascites. The patients were diagnosed with liver cirrhosis based on the results of liver biopsy and/or imaging studies (computed tomography and USA). One hundred and four volunteers (48 males and 56 females) were also evaluated as control.

For all patients, 5-500 kHz multifrequency BIA was performed using InBody 3.0 (Exercise Physiology USA and Biospace Co., Ltd.)[11]. On the same day, they underwent general laboratory examination for albumin, bilirubin, cholesterol, aspartate aminotransaminase (AST), alanine aminotransaminase (ALT), and blood platelet count. The patients and control volunteers fasted overnight prior to the laboratory examinations. FFM was calculated as described previously[10,11]. After measurement of FFM, serum biochemistry assays were performed every month on an outpatient basis. The average observation period was 15.4 mo, ranging from 12 to 24 mo.

Table 1 Characteristics of the 57 patients at base line (mean±SD)

Variables	Male	Female	All
n	26	31	57
Age (yr)	62.0±11.4	66.0±10.0	64.2±10.8
Etiology (HCV/HBV/Alcohol/PBC)	23/2/1/0	27/2/1/1	50/4/2/1
Child-Pugh (A/B/C)	23/3/0	29/2/0	52/5/0
BMI	23.7±2.2	22.0±2.7	22.8±2.6
Albumin (g/dL)	3.38±0.49	3.57±0.47	3.48±0.48
Bilirubin (mg/dL)	1.31±0.64	1.09±0.49	1.19±0.57
AST (U/L)	87.2±71.7	76.0±55.1	81.1±62.9
ALT (U/L)	77.7±72.3	64.9±70.1	70.7±70.8
Cholesterol (mg/dL)	155.3±38.2	156.3±27.3	155.9±32.3
ChE (mg/dL)	83.0±32.2	92.0±38.9	88.0±36.0
Platelet ($\times 10^4$/L)	9.1±3.8	10.9±6.5	10.1±5.5

Statistical analysis

The results of laboratory data are shown as mean±SD. Differences in the average and dispersion of FFM and rFFM between males and females were confirmed by *t*-test and f-test. Stepwise multivariate linear regression analysis was performed to confirm the significant predictive factors for albumin stability using variables such as rFFM, albumin, bilirubin, cholesterol, ALT, and blood platelet count.

RESULTS

Standard FFM of volunteers

In order to evaluate the relationship between FFM and height, 48 male and 56 female volunteers, whose body mass index (BMI) ranged from 20 to 24, underwent multifrequency BMI. As shown in Figure 1, there was a good correlation between FFM and height for males and females, and the standard FFM could be calculated as below. Male: standard FFM (kg) = 0.565×height (cm) - 43.261; Female: standard FFM (kg) = 0.677×height (cm) - 66.084.

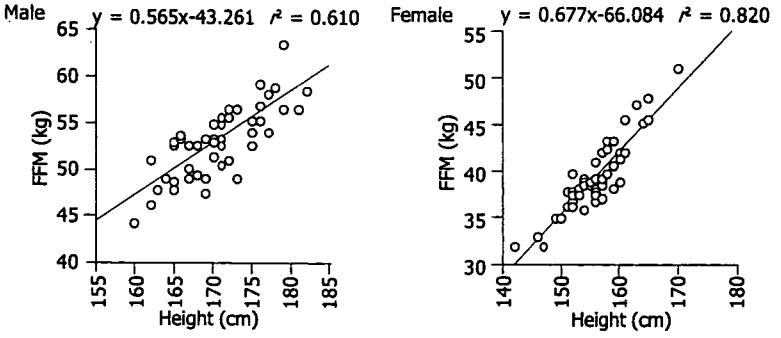

Figure 1 Correlation between fat-free mass (FFM) and height in volunteer males (right panel) and females (left panel). There was a significant positive correlation between FFM and height in each sex (male: $r = 0.781$, $P<0.0001$; female: $r = 0.906$, $P<0.0001$).

Calculation of relative FFM

According to the regression formula above, expected FFM was calculated using the height for each patient. Then relative FFM (rFFM) was then calculated as follows. rFFM (%) = (measured FFM/standard FFM) ×100. In short, rFFM represents the ratio between the FFM of a patient and that of a normal control with an average structure and the same height as the patient.

The left columns in Figure 2 show the FFM distributions of cirrhotic patients, and there was a significant difference in dispersion between males and females ($P<0.001$). In calculating rFFM for the patients (right columns of Figure 2), the dispersion did not differ significantly between males and females. Therefore, it was possible to compare rFFM of patients regardless of their sex differences.

SD-albumin and -albumin

Serum albumin levels were measured for all patients every month. Using the monthly results, standard deviation was calculated for all patients (SD-albumin). The difference between the maximum and minimum results during the entire observation period was also calculated (albumin). Both SD-albumin and -albumin were considered to be indications of the stability of hepatic synthetic function.

Comparison of rFFM and SD-albumin, or rFFM and -albumin for all patients, showed no significant correlations. However, when the analysis was limited to patients with average ALT levels over 50 U/L, we found significant correlations between rFFM and SD-albumin, and between rFFM and -albumin (Figure 3).

Multiple regression analysis

It is reasonable that rFFM correlates significantly with SD-albumin and -albumin in patients with high ALT levels, since the demand for amino acids would be increased under conditions in which hepatocytes are regenerating. However,

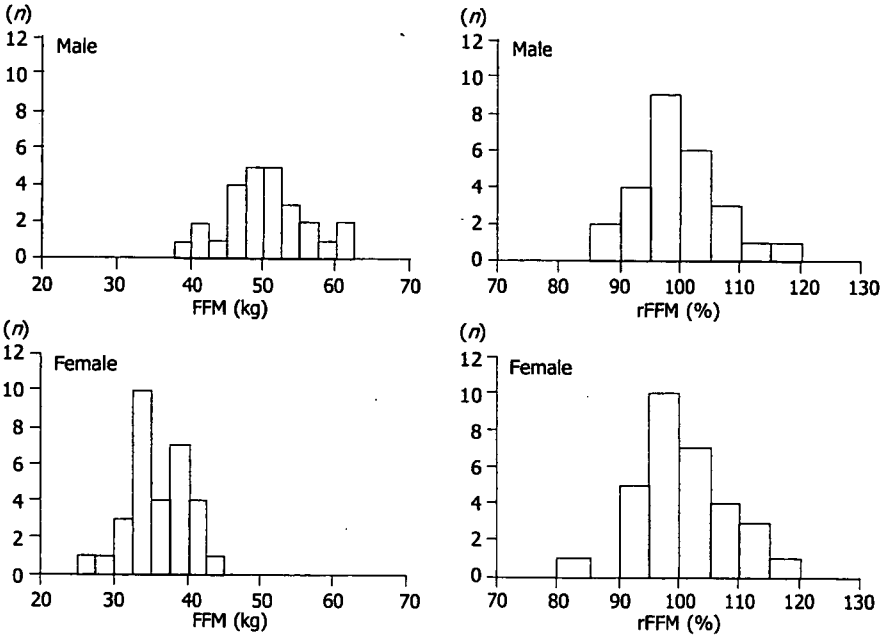

Figure 2 Distribution of fat-free mass (FFM) and relative FFM (rFFM) in cirrhotic patients. In FFM, the distributions among the sexes differed significantly (left two columns) ($P<0.001$). In contrast, when rFFM was applied for the same patients (right two columns); the dispersion did not differ significantly.

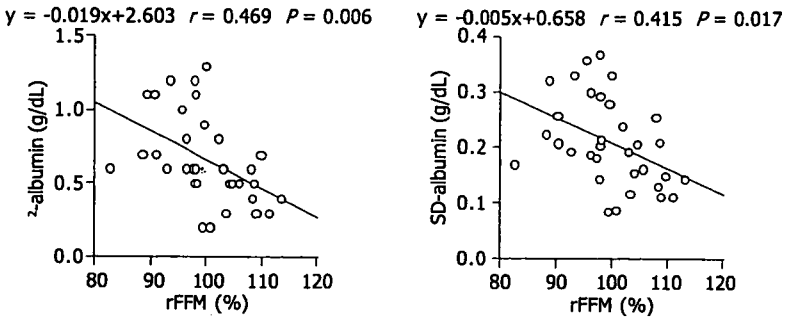

Figure 3 Correlation between rFFM with -albumin (right panel) and SD-albumin (left panel). In patients with average ALT levels >50 U/L, there was a significant correlation of rFFM with SD-albumin ($r = 0.469$, $P = 0.006$) and with -albumin ($r = 0.415$, $P = 0.017$).

other factors, such as the hepatic functional reserve or nutritional status at the time of rFFM measurement, might also influence SD-albumin and -albumin.

To investigate this possibility, we performed stepwise regression analysis using variables such as rFFM, platelet number, and the serum levels of cholesterol, chorine esterase, albumin, bilirubin and ALT. This analysis revealed that rFFM and ALT were independent predictors for both -albumin and SD-albumin (Table 2).

Table 2 Univariate analysis of -albumin and SD-albumin, and multivariate analysis using potential univariate predictors

	vs -Albumin				
	Univariate analysis			Multivariate analysis	
	R2	F	*P*	F	*P*
rFFM	0.094267	5.6202	0.0214	6.0402	0.0173
Albumin	0.016847	0.9254	0.3404	-	-
Bilirubin	0.017005	0.9342	0.3381	-	-
ALT	0.08064	4.7365	0.0339	5.0355	0.0290
Cholesterol	0.009282	0.4965	0.4841	-	-
Platelet	0.021799	1.1811	0.2821	-	-
	vs SD-Albumin				
	Univariate analysis			Multivariate analysis	
	R2	F	*P*	F	*P*
rFFM	0.06456	3.7268	0.0488	3.8602	0.0447
Albumin	0.02243	1.2390	0.2706	-	-
Bilirubin	0.003762	0.2039	0.6534	-	-
ALT	0.072818	4.2410	0.0443	4.3666	0.0415
Cholesterol	0.005361	0.2857	0.5952	-	-
Platelet	0.02186	1.1845	0.2814	-	-

DISCUSSION

It has been widely accepted that the determination of body composition is useful for evaluating nutritional status. However, traditional methods, such as skinfold measurement, are easy to perform but lack accuracy and reproducibility. On the other hand, other methods such as tracer dilution, neutron activation analysis and DEXA require sophisticated equipments and skilled technique. In recent years, BIA has emerged as a simple and reproducible method that can be used for the evaluation of FFM. Several reports have shown that the results of BMI correlate well with those of other methods[12,13].

Regardless of the method used for the analysis of body composition, sex differences have been a substantial obstacle. The results of measurements in males and females cannot be readily compared since their average body composition values differ significantly. To solve the problem, we applied a new factor, rFFM (= [measured FFM/standard FFM] ×100%). Using this new variable, we could compare the FFM results of all patients, regardless of their sex. Although we used the formula derived from the correlation between the FFM and height of the controls in calculating rFFM, the average body compositions are known to differ by race[14,15]. Therefore, the controls for standard curves should be prepared for each individual study.

Our results revealed that rFFM correlated with -albumin and SD-albumin in patients with high ALT levels, which indicated that muscle volumes would be one of the most important factors for maintaining the serum albumin level in active cirrhosis. This result seems reasonable since muscle protein serves as an amino acid pool. It should be noted that the stability of serum albumin levels is important not only for predicting the prognosis of patients with liver cirrhosis, but also to ensure a high quality of life. Serum albumin accounts for the colloid osmotic pressure of plasma; therefore hypoalbuminemia causes ascites, edema, and a reduction of circulating plasma volume, which would exacerbate hepatic failure.

In order to maintain or improve muscle volume, two interventions should be considered namely, amino acid supplementation and exercise. Cirrhotic patients often suffer from negative energy balance even at an early stage of the disease, characterized predominantly by protein deficiency and it is well known that they also have reduced plasma branched-chain amino acid (BCAA). Recent studies demonstrated the efficacy of branched-chain amino acid supplementation in improving the hypoalbuminemia of cirrhotic patients[7,8]. In the present study, none of the patients had received BCAA-supplementation because decompensated patients were excluded. Theoretically, BCAA-supplementation could inhibit muscle catabolism and contribute to the maintenance of muscle volume.

Regarding the usefulness of exercise for cirrhotic patients, most reports have focused on decompensated patients with ascites. Salo *et al.*[16], noted that moderate physical exercise caused a marked impairment in the renal function of patients with ascites as well as marked stimulation of vasoconstrictor systems, whereas Garcia-Pagan *et al.*[17], indicated that moderate exercise increased portal pressure and might therefore increase the risk of variceal bleeding in patients with esophageal varices. Although the contribution of exercise to the prognosis of cirrhotic patients remains unclear, we believe that exercise at the compensated stage would be useful for maintaining muscle volume which could delay the emergence of symptoms such as ascites and edema that accompany the progression of cirrhosis. Once ascites emerge in a cirrhotic patient, abdominal fullness causes appetite loss, which induces further hypoalbuminemia and reduction of hepatic blood flow. To avoid such an injurious cycle, it is important to prevent hypoalbuminemia at the compensated stage. Taken together, the evidence suggests that the periodic evaluation of body composition using BAI, in addition to the physician's recommendation for exercise, would be useful for improving the prognosis of compensated cirrhotic patients.

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Clinical Studies

A multi-step, incremental expansion method for radio frequency ablation: optimization of the procedure to prevent increases in intra-tumor pressure and to reduce the ablation time

Kotoh K, Nakamuta M, Morizono S, Kohjima M, Arimura E, Fukushima M, Enjoji M, Sakai H, Nawata H. A multi-step, incremental expansion method for radio frequency ablation: optimization of the procedure to prevent increases in intra-tumor pressure and to reduce the ablation time. Liver International: 2005: 25: 542–547. © Blackwell Munksgaard 2005

Abstract: *Background/Aims:* Radio frequency ablation (RFA) has been accepted clinically as a useful local treatment for hepatocellular carcinoma (HCC). However, intra-hepatic recurrence after RFA has been reported. We initially hypothesized that recurrence was attributable to increases in intra-tumor pressure during RFA, and we subsequently measured the pressure and optimized the procedure. *Methods:* A block of pig liver sealed in a rigid plastic case was used as a model of an HCC tumor with a capsule. We compared the pressure between a single-step full expansion of the needle (single-step method) and incremental, stepwise expansion (multi-step method), and evaluated the effect of varying the electrical power. Finally, we performed a preliminary comparison of the ablation times for these methods in HCC cases. *Results:* The multi-step method resulted in a significantly lower pressure and shorter total ablation time than the single-step method. Furthermore, incremental expansion in 10 steps resulted in a lower pressure and shorter ablation time than four steps. Seventy W-ablation resulted in a lower pressure and shorter time than 30- or 50 W-ablation. In HCC cases, the multiple-step method had a significantly shorter ablation time than the single-step method. *Conclusion:* The multi-step method can be recommended to reduce the ablation time, and suppress the increase in pressure.

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Key words: ablation time – hepatocellular carcinoma (HCC) – intra-tumor pressure – multi-step expansion – radio frequency ablation (RFA) – single-step expansion

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Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and usually is associated with hepatitis B virus (HBV) or hepatitis C virus (HCV) infection. In Japan, over 80% of HCC patients suffer from HCV-induced liver injury and most have underlying liver cirrhosis (1). Consequently, surgical resection is impossible in many cases of HCC because of a poor hepatic functional reserve. Under such conditions, percutaneous ethanol injection therapy (PEIT) has been used widely for the treatment of unresectable HCC (2). Many reports showed that the efficacy of PEIT for small HCC tumors was comparable with that of hepatic resection. As we demonstrated previously, however, PEIT demands multiple sessions to achieve complete necrosis, resulting in protracted hospitalization (3). Furthermore, many patients suffer from local recurrence after PEIT (4, 5). Such recurrence is considered to be attributable to intra-tumor septa that prevent the injected ethanol from infiltrating the entire tumor. We believe that local recurrence after PEIT should be prevented as much as possible because it is one of the most important negative prognostic factors for HCC patients (unpublished data).

Over the last several years, radio frequency ablation (RFA) has become a popular alternative

Abbreviations: RFA, radio frequency ablation; HCC, hepatocellular carcinoma; PEIT, percutaneous ethanol injection therapy.

to PEIT because it requires only one session to achieve complete necrosis (6, 7). Although RFA was initially expected to decrease the incidence of local recurrence, recent reports indicate that local recurrence after RFA is not uncommon (8–12). Furthermore, in some cases, the recurrence was rapid and vessel-invasive (13, 14). The recurrence in RFA seems unusual because thermal ablation is considered to achieve complete necrosis readily, regardless of intra-tumor septa.

Because explosive sounds are often heard during the RFA procedure, it is feared that rapid heating of a tumor may lead to an unpredicted increase in internal pressure and this might cause the dislodgement and scattering of malignant cells around the ablated tumor; however, there is no direct evidence that the high internal pressure RFA attributed to local recurrence. To confirm this hypothesis, we measured the intra-hepatic pressure during RFA using a model comprising a liver block sealed within a rigid plastic case. Although we neglected the influence of blood flow in this model, it was considered to be similar to a well-encapsulated HCC tumor. In addition to comparing the various conditions for ablation, we tried to optimize the procedure to reduce ablation time and to avoid increases in pressure.

Materials and methods

Measurement of ablation time and pressure *in vitro* model

Pig livers were prepared for the ablation studies 6 h after sacrifice. Two blocks of liver tissue ($5 \times 5 \times 4\,cm^3$) were cut and packed into a rigid $5 \times 5 \times 8\,cm^3$ plastic case with a pressure sensor (model P303-01, M0101D; SSK Co., Ltd., Tokyo, Japan) mounted at one end (Fig. 1). The case containing the pig liver was cooled on ice for 15 min before ablation in order to maintain constant conditions. We used a LeVeen® needle, an expansion-type electrode with 10 tines in an umbrella pattern, as the device for RFA (3.0 cm type; Boston Scientific Corporation, Natick, MA, USA) and supplied the electric current from a generator (model RF2000®: Boston Scientific Corporation). In this apparatus, the resulting increase in tissue resistance to current flow (impedance) is measured in ohms, and when the impedance reaches a programmed end point, the current is automatically shut off. At the programmed end point of the ablation (roll-off), clinically sufficient necrosis is achieved.

As shown in Fig. 1, the electrode needle was inserted from the opposite end of the apparatus to the pressure sensor, until the tip of the needle

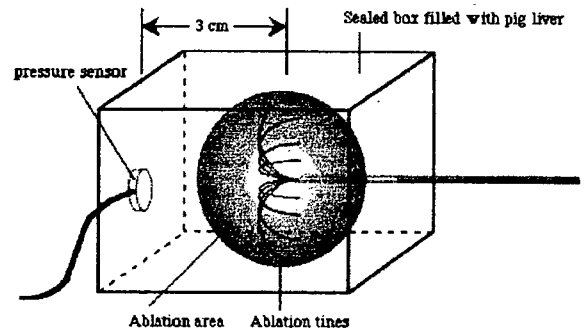

Fig. 1. The system for measuring pressure during ablation is shown. A block of pig liver was compactly packed into a rigid plastic case. A pressure sensor was located at the one end of the case, and the electrode needle was inserted from the opposite end until the tip of the needle was 3 cm from the sensor.

reached 3 cm from the sensor. For analysis of the single-step method, the pressure was measured every 20 s until complete ablation (roll-off) was achieved. The pressure at the programmed end point was also measured. The multi-step method also was examined using this pressure-measuring system. The stepwise expansion was performed using two different procedures: four steps and 10 steps. In the four-step method, a quarter of the length of the electrode tines was expanded for the first step and the current was delivered until roll-off was achieved. Next, half the length of the tines was expanded for the second-step and the current was supplied. For the third and final steps, the tines were expanded to three quarters of their full-length. Similarly, in the 10-step method, the tines were expanded by one-tenth of their length at each step, up to complete expansion for the final-step, and roll-off was achieved at each step. In the 10-step method, the ablation was performed at 30, 50 and 70 W, and the time to achieve roll-off and the pressure at the programmed end point were recorded at each step. In order to confirm the change in cell density under the ablation pressure, the histological findings in the pig liver were examined by hematoxylin and eosin (H&E) staining after single- and four-step ablation.

Measurement of ablation time in clinical HCC cases

We performed a preliminary comparison of ablation times for the single- and multi-step methods in 20 HCC cases with liver cirrhosis (Table 1). For the single-step method ($n = 10$), ablation was started at 50 W, and the electrical power was increased by 10 W/min in the subsequent ablation until 90 W was reached. For the multi-step method ($n = 10$), ablation was started at 50 W. The electrical power was increased to 70 W at the fifth-step and to 90 W at the final-step. Tumor location, tumor size, and the area ablated by

Table 1. Comparison of clinical backgrounds, ablation time, and RFA-treated area size between the single-step method and the multi-step method in clinical HCC cases

	Single-step	Multi-step
n	10	10
Sex (M/F)	8/2	7/3
Age	55.6 ± 2.9	57.2 ± 2.5
Child A/Child B	8/2	9/1
Tumor location (rt./lt. lobe)	9/1	9/1
Tumor size (mm)	22.1 ± 2.1	21.1 ± 1.6
Ablation time (s)	21.4 ± 1.7	10.7 ± 2.3*
Ablated area (mm)	32.5 ± 2.4	33.6 ± 2.2

Plus-minus values are means ± SEM. Tumor size and the area ablated by RFA are expressed as the diameter (mm). χ^2-test and non-paired *t*-test showed no significant background difference between the single-step method and multi-step methods. RFA, radio frequency ablation; HCC, hepatocellular carcinoma; rt., right; lt., left; M, male; F, female. *$P<0.0001$.

RFA were determined by computed tomography examination.

Statistical analysis

All measurements were performed three times, and the results are shown as mean ± SEM. Statistical comparisons for the cumulative ablation time and the pressure at the programmed end point *in vitro* were made using ANOVA and Scheffe's test using Statview software (SAS Institute, Cary, NC, USA). Statistical comparisons for tumor size, ablation time, and the area ablated by RFA were made using non-paired *t*-test with Statview software.

Results

To determine the pressure during ablation and the time for roll-off in the single-step method, the electrode tines were expanded fully before ablation at 50 W and the pressure was measured every 20 s (Fig. 2, upper panel). In this method, the pressure increased rapidly and markedly after the ablation, it took 261.3 ± 4.67 s (mean ± SEM) to achieve roll-off and the pressure at the end point of the ablation was 837.1 ± 21.3 kPa, which is equivalent to the tire pressure of a vehicle.

In contrast to the single-step method, multi-step ablation (four- or 10-step expansion) at 50 W required a much shorter time to achieve roll-off at each step, and the pressure at the end point of the ablation also was kept very low for all steps (Fig. 2). Although the pressure at each ablation increased with the expansion of the electrode tines, the elevation of the pressure was quite gradual. Comparing four- and 10-step ablation, the latter resulted in a lower pressure and shorter time to achieve complete necrosis in the incremental steps.

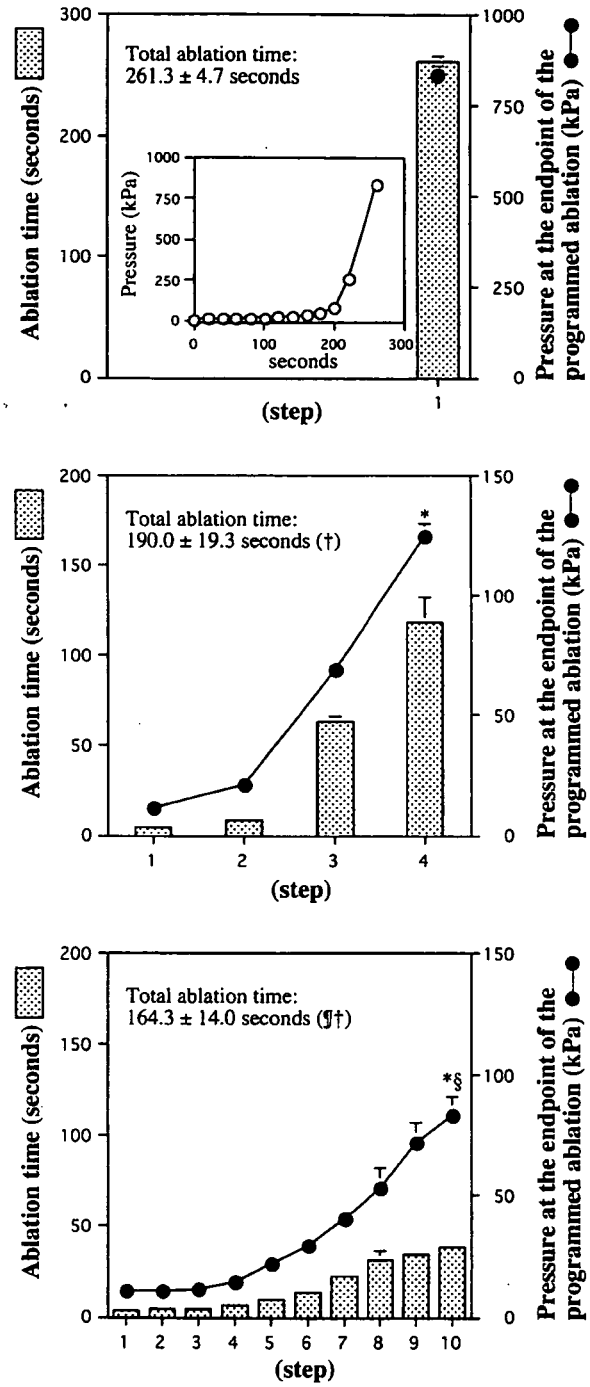

Fig. 2. The ablation time (dotted bar) and the pressure at the programmed end point (closed circle) are shown for each step. In all methods, single-step (upper panel), four-step (middle panel), and 10-step (lower panel), the ablation was performed at 50 W. The change in pressure during single-step ablation is also shown in the upper panel. The pressure was rapidly elevated after the ablation time over 200 s. The single-step method resulted in a significantly higher pressure and longer ablation time than the multi-step methods. For the multi-step methods, the 10-step ablation was shorter and finished with lower pressure than the four-step method. (*$P<0.001$ vs. single-step, §$P<0.05$ vs. four-step, †$P<0.01$ vs. single-step, ¶0.05 vs. four-step).

Comparing the cumulative ablation time of the entire process, that for the multi-step ablation was significantly shorter than that for the one-step method (Fig. 2). For the multi-step methods, the 10-step ablation time was significantly shorter than that of four-step ablation (Fig. 2).

We next evaluated the effects of the electrical power on both the time for ablation and pressure. Thirty W-ablation using the 10-step method resulted in a longer time but lower pressure to achieve roll-off in each step compared with 50 W-ablation (Fig. 3). On the other hand, 70 W-ablation using the 10-step method resulted in not only a shorter time, but also a lower pressure than 30- or 50 W-ablation (Fig. 3). Overall, 50 W-ablation resulted in the highest pressure and 30 W-ablation required the longest time to complete all steps (Fig. 3). In contrast, 70 W-ablation resulted in the lowest pressure and shortest time (Fig. 3).

Histological examination after multi-step ablation (four-step method) showed that the cell density differed between the outer and the inner portions of the ablated region (Fig. 4). That is, the cell density near the top of the needle was higher than that close to the margin of the region of ablation, whereas the difference in cell density was not seen after one-step ablation (Fig. 4).

In a preliminary clinical trial, we compared ablation time between the multi-step method ($n = 10$) and the single-step method ($n = 10$). The multi-step method showed a significantly shorter ablation time than the single-step method ($P < 0.0001$) (Table 1), and there was no significant difference in the area ablated by radio frequency. There were no adverse events during treatment with either of the methods.

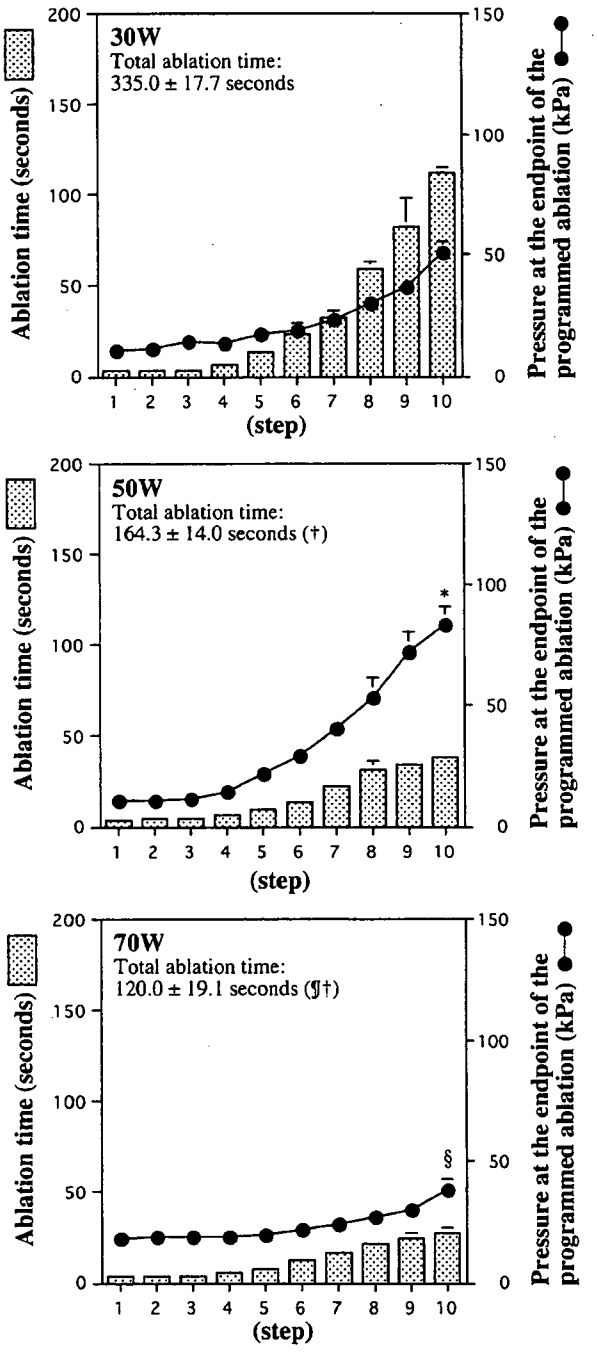

Fig. 3. The 10-step ablation was performed with varied electrical power. The ablation at 30 W took the longest, and that at 50 W resulted in the highest pressure; whereas the ablation at 70 W resulted in the shortest time and the lowest pressure. (*$P < 0.05$ vs. 30 W, §$P < 0.01$ vs. 50 W, †$P < 0.001$ vs. 30 W, ¶0.05 vs. 50 W).

Discussion

In this study, we showed that varying the setting of the RFA device could influence the ablation time and the pressure during ablation. For clinical application, we should aim for the shortest ablation time in order to reduce the patient's discomfort during treatment, and also to decrease the pressure during ablation to prevent the possibility of intra-hepatic metastasis. During RFA treatment of hepatic tumors, the pressure caused by ablation would not reach the high levels observed in this study, because the increased pressure could escape through the arteries and veins penetrating the tumor. However, a situation similar to our model could occur in a tumor with poor arterial flow and a thick capsule. The increased fibrosis in cirrhotic patients might have the same effect as a tumor capsule, containing the pressure produced by ablation.

The multi-step method required a significantly shorter ablation time and resulted in a much lower pressure during ablation than the single-step method. The difference in the ablation time is

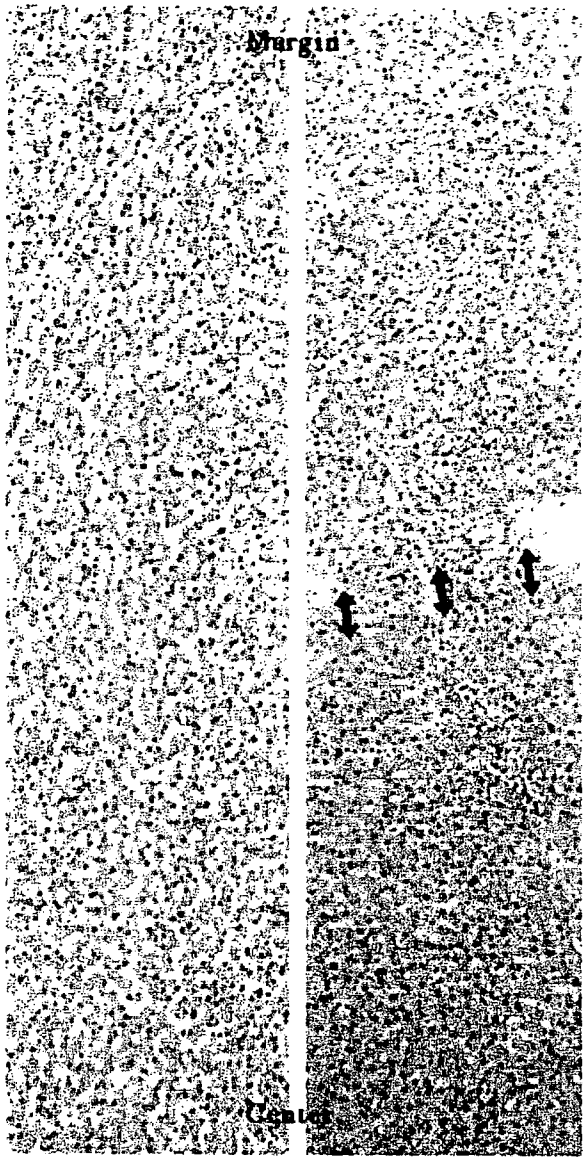

Fig. 4. Histological findings are shown after single-step (left panel) and four-step (right panel) ablation. The cell density near the center of the region of ablation was higher than that close to the margin in the four-step ablation, whereas this difference was not seen after single-step ablation. Arrows indicate the margin between the high and low cell density regions.

thought to be attributable to the efficiency of energy use. That is, the single-step procedure requires continuous ablation until the entire targeted region becomes necrotic. Furthermore, the extra-tumorous tissue, in addition to the tumor, is heated, resulting in wasted energy. In contrast, in the multi-step procedure, the region of ablation is limited in each step, preventing unnecessary energy loss.

That difference in the region of ablation may also explain the inequality in pressure during

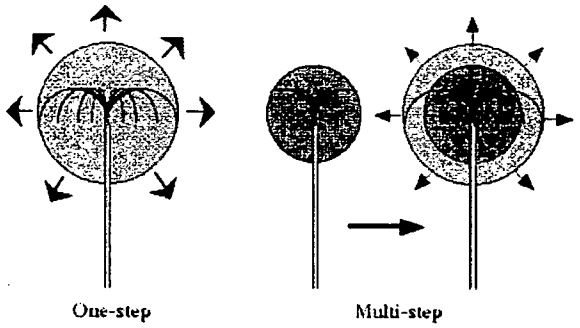

Fig. 5. A possible explanation for the mechanism of pressure reduction in the multi-step procedure, according to the histological findings in Fig. 4. Arrows indicate the pressure during RFA. In the single-step procedure, the entire ablated region is simultaneously heated, and the high pressure generated is forced outside the ablated region. In contrast, in multi-step ablation, some of the pressure caused by ablation during the second and subsequent steps could escape into the internal region, resulting in lower pressure than in the single-step ablation.

ablation. In the single-step procedure, all ablated cells in the entire targeted region would expand simultaneously to generate high pressure, while the ablation of a limited region in the multi-step procedure would result in a lower level of intra-tumor pressure. Furthermore, histological findings indicated another mechanism of pressure reduction in the multi-step procedure. After multi-step ablation, the cell density of the internal region was higher than that of the outer region. We speculate that this indicates that some of the pressure caused by ablation during the second and subsequent steps could escape into the internal region, which was ablated in previous steps, and the cells within it would become friable (Fig. 5).

Considering the mechanism of the successive effects during the multi-step procedures, it is inevitable that the 10-step procedure is superior to the four-step, because the greater the number of steps used for ablation, the lower the pressure and the shorter the time required. Because the procedure becomes increasingly complicated as more steps are added, we propose that an eight- to 10-step procedure is efficient and effective in clinical application.

The electrical power used for RFA is another parameter that should be carefully considered. According to the manual of the device, it is recommended that ablation should be started at 50 W and the electrical power should be increased by 10 W/min in the subsequent ablation until 90 W is reached. The reason the ablation is started at low power is essentially to decrease the pain of the patient. However, the relationship between the electrical power and the intra-tumor pressure, and the duration of the ablation, has not been clarified. Our results showed that the

ablation time became shorter with the increase in the electrical power, whereas the intra-tissue pressure increased at 50 W but subsequently decreased at 70 W (Fig. 3). The inverse relationship between the duration and the electrical power is easily understood, but the decreased pressure at high power seems paradoxical. One possible explanation of this paradox is that the ablation with very high electrical power may achieve complete necrosis rapidly, before the tissue temperature around the targeted region increases, so that the total pressure would be kept at a relatively low level. If we accept this explanation, the ablation time could be shortened and the intra-tumor pressure would decrease with an increase in the electrical power, without limitation. But we must remember that ablation at high power induces severe pain. Therefore, the upper limit of the electrical power should be determined according to the suffering of the patient and the extent of the pressure reduction. We are now applying our multi-step method in clinical RFA treatment, and preliminary results indicate that the multi-step method had significantly shorter ablation times than the single-step method (Table 1). We plan to further evaluate the appropriateness of this procedure for clinical use, and confirm whether the intra-tumor pressure created by the sudden heating of RFA contributes to the spreading or local recurrence of HCC.

In this study, we showed that the standard, single-step method might entail a risk of extreme increases in intra-tumor pressure under some conditions, which could result in scattering of intra-hepatic metastasis. Our model is artificial and without blood flow, and a system with perfusion using an entire liver would be required to simulate the condition of a living liver more accurately. Finally, we emphasize that intra-tumor pressure could be greatly reduced by a simple modification of the use of the standard device, simply by adopting an incremental, multi-step method. We believe that this multi-step method should be applied as a standard clinical procedure for RFA.

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Splenic large B-cell lymphoma in patients with hepatitis C virus infection☆

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Summary Hepatitis virus infection, especially type C (hepatitis C virus [HCV]), has been suggested to be one of the important pathogenetic factors for low- and high-grade B-cell lymphoma, including splenic marginal zone lymphoma (SMZL), in southern Europe. Here, we analyzed the incidences of HCV and hepatitis B virus (HBV) infections, and the clinicopathologic features in 29 cases of splenic diffuse large B-cell lymphoma (DLBCL), 10 SMZL, 3 splenic mantle cell lymphoma, 1 hairy cell leukemia, 13 B-chronic lymphocytic leukemia, and 12 hepatosplenic T-cell and natural killer cell lymphoma. Fifteen (51.7%) splenic DLBCL cases were HCV antibody–positive, and another 6 (20.7%) had the HBsAg. The incidence of each was significantly ($P < .01$) higher than those of HCV (9.3%) and HBV (1.9%) infections in 54 node-based DLBCL cases. Four examined HCV-positive DLBCL cases showed no type II cryoglobulinemia. HCV RNA was detected in fresh tumor tissues from 6 of 7 examined DLBCL cases, and HBV DNA was present in another 2, as evaluated by real-time polymerase chain reaction. Immunohistologically, tumor cells in 5 of 7 examined DLBCL cases showed intracytoplasmic reactions for HCV NS3 and E2 proteins and the viral receptor CD81. Of 6 cases, 2 showed an intranuclear reaction for the HBV surface protein. By Southern blot analysis, no rearrangement of the *Bcl2* gene was detected in the tumor tissue of 7 HCV-positive DLBCL cases. For the other types of malignant lymphoma, 1 case each of SMZL (10%) and hepatosplenic T-cell and

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natural killer cell lymphoma (8.3%) showed HCV infection. In conclusion, persistent human hepatitis virus infections, especially HCV, may play an important role in the tumorigenesis of splenic DLBCL in Japan.

1. Introduction

Hepatitis C virus (HCV) infection may be involved in the pathogenesis of type II (monoclonal IgM and polyclonal IgG) cryoglobulinemia (CG) and low- and high-grade B-cell malignant lymphoma (ML) in southern Europe [1,2]. De Vita et al [3] reported that B-cell ML in HCV-positive patients presented extranodal localization frequently in the bone marrow and major salivary glands and occasionally in the liver and spleen and histologically showed lymphoplasmacytic lymphoma (LPL) and diffuse large B-cell lymphoma (DLBCL). Serology of HCV infection was found in 11 (35%) of 31 Italian cases of splenic marginal zone lymphoma (SMZL) [4]. Hermine et al [5] further reported 9 French cases of HCV-positive SMZL with type II CG. Because these 9 cases showed loss of HCV RNA in their sera and complete remission of ML after interferon (IFN) α and ribavirin treatments, the authors suggested that HCV infection plays a role in the lymphomagenesis of SMZL. Japan is also an endemic area for HCV infection, and HCV-related ML cases have been reported [6,7]. Although SMZL is rare among the total ML cases in Japan [8], we examined the relationships between the histological types of spleen-involving ML and hepatitis virus (HV) infection. We found a high incidence of HCV infection in splenic DLBCL cases and a low incidence in SMZL. We discuss the cause of the strikingly different histological types of splenic B-cell ML with HCV infection between Japanese and southern European patients. Sansonno et al [9] demonstrated HCV RNA in reactive lymph nodes and neoplastic tumor tissues of B-cell ML by reverse transcription–polymerase chain reaction (PCR) and immunohistologically detected the HCV core and NS3- and NS4-related proteins, C100, c22, and c33, in the tumor tissues of 3 of 12 HCV-positive B-cell ML cases. The HCV-encoded NS3 protease and helicase have central roles in the viral replication cycle [10]. The E2 protein of HCV binds the CD81 receptor in B lymphocytes, and internalization of the virus may activate the signaling pathway and induce lymphomagenesis [11]. We found the presence of HCV RNA in the tumor tissues of ML by real-time PCR and also detected HCV NS3 and E2 proteins plus CD81 in the lymphoma cells. The etiologic role of HCV infection is discussed.

It is known that *Bcl2* is an important apoptosis inhibitor for B cells and their neoplasm and that *Bcl2* translocation is frequently detected by PCR in the peripheral blood mononuclear cells (PBMCs) of patients with HCV infection [12]. *Bcl2* gene expression may reflect a premalignant state of B cells with HCV infection. We examined for any rearrangements of the *Bcl2* and *Bcl6* genes and proteins in the tumor tissues.

Hepatitis B virus (HBV) may also play an important role in lymphomagenesis in B-cell ML [13]. The influence of HBV infection in splenic ML is discussed.

2. Patients and methods

We investigated 68 patients with ML, primarily involving the spleen. Thirteen cases of B-chronic lymphocytic leukemia (CLL) and 12 of hepatosplenic T-cell lymphoma and aggressive natural killer (NK) cell leukemia/lymphoma (HST/NKL), which mainly involved the spleen, were included. The clinical and laboratory findings, treatments, and prognoses were examined for each hospital. Fifty-four node-based DLBCL cases and 445 colon cancer cases admitted to the National Kyushu Medical Center were selected for examination of their HCV and HBV infections as a control. Precipitated serum cryoglobulin was measured using a micro–TP-AR kit (Wako Chemical Industries, Osaka, Japan), and the immunoglobulin composition was determined by immunoelectrophoresis.

2.1. Examination of HCV and HBV infections

Serum samples were screened for HCV infection by assaying the amount of anti-HCV antibodies using a second-generation enzyme-linked immunosorbent assay technique. The HCV genotype was determined by a PCR-based technique (SMI Test HCV Genotyping Kit; Sumitomo Metals, Osaka, Japan), which identified 4 genotypes (1a, 1b, 2a, and 2b) according to the classification of Simmonds et al [14]. HBV surface and envelope antigens were also screened with an enzyme-linked immunosorbent assay kit (Abbott Laboratories, Abbott Park, Ill).

2.2. Quantitative real-time PCR for HCV RNA and HBV DNA

Fresh frozen tumor tissues of 8 HCV-positive splenic ML cases and reactive lymph nodes of 3 HCV-positive cases were examined. Total RNA was extracted from 10-mg fresh frozen tumor tissues from HCV-positive ML patients using the isogen-LS RNA extraction system (Nipon Gene, Tokyo, Japan). Purified RNA was suspended in 20 μL of diethyl pyrocarbonate–treated water containing 10 mmol/L dithiothreitol and 200 U/mL ribonuclease inhibitor. The RNA

Table 1 Antibodies used in immunostaining and detection kit

Antibody	Clone	CD no.	Source
CD3	PS1	CD3ε	Novocastra
CD5	4C7	CD5	Novocastra
CD10	56C6	CD10	Novocastra
CD20	L26	CD20cy	Dako
IL-2 receptor	4C9	CD25	Novocastra
CD103	2G5	CD103	Immunotech
Bcl1	5D4		IBL
Bcl2	124		Dako
Bcl6	P1F6		Novocastra
NCAM1	123C3.D5	CD56	NeoMarkers
TIA1	NS/1-AG4		Coulter
CD81	1D6	CD81	Novocastra
HCV NS3	MMM33		Novocastra
HCV E2			ViroStat
HBs Ag	ZCH16		Nichirei
HHV-8 LNA	13B10		Novocastra
In situ hybridization and detection kit for EBER (Rembrandt)			Kreateck

TIA1, T-cell intercellular antigen; LNA, latent nuclear antigen. Company locations are as follows: Novocastra, Newcastle upon Tyne, England; Dako, Carpinteria, CA; Immunotech, Marseille, France; IBL, Takasaki, Japan; Neomakers, Fremont, CA; Coulter, Hialeah, FL; ViroStat, Portland, ME; Nichirei, Tokyo, Japan; Kreateck, Amsterdam, Netherlands.

solution was used for real-time PCR, which was performed using a TaqMan EZ RT-PCR Core Reagent kit and an ABI 7700 sequence detector system (Perkin Elmer, Foster City, Calif) [15]. HBV DNA was extracted from 10-mg fresh frozen tumor tissues and was determined by real-time PCR as described previously [16].

2.3. Histology, immunohistology, and in situ hybridization

Tissue specimens were fixed with 20% formalin, embedded in paraffin, and stained with hematoxylin-eosin solution. The histological diagnosis of the first biopsy and resected samples and the primary sites were decided according to the World Health Organization classification and its criteria [17]. For immunohistology, a battery of monoclonal antibodies (Table 1) was applied to formalin-fixed samples using the Chemmate Envision method (Dako, Carpinteria, Calif) and an alkaline phosphatase–conjugated avidin-biotin method (Vector, Burlingame, Calif). Human herpesvirus (HHV) 8 infection was checked by immunohistology for the HHV-8–encoded protein latent nuclear antigen (LNA). To detect Epstein-Barr virus (EBV) infection, paraffin sections were hybridized in a solution of 50% formamide-containing digoxigenin/biotin-labeled EBV-encoded RNA (EBER) oligonucleotides (Rembrandt Kit, Kreateck, Amsterdam, The Netherlands).

2.4. Detection of the Bcl2 and Bcl6 genes

Fresh frozen tumor tissues from 8 HCV-positive B-cell ML cases and reactive lymph nodes of 2 HCV-positive cases were examined. Rearrangements of the *Bcl2* and *Bcl6* genes were analyzed using *Bcl2* MRB and mcr probes, and *Bcl6* LAZ3-A and LAZ3-2 probes, respectively. Five-microgram aliquots of genomic DNA were digested with *Bam*HI or *Hin*dIII restriction endonucleases, electrophoresed in 0.8% or 1% agarose gels, denatured with alkali, neutralized, and then transferred to nitrocellulose filters. The filters were hybridized in 50% formamide/3× standard citrate buffer at 37°C with DNA probes that had been labeled with phosphorus 32 using a Random Primed DNA labeling kit (Boehringer Mannheim, Mannheim, Germany).

2.5. Statistical analyses

To confirm differences between 2 groups of lymphoma patients, univariate analyses by the χ^2 and Fisher tests were performed.

Table 2 Clinical findings of ML, mainly involving spleen

	Diffuse large B-cell ML	Marginal zone	Mantle cell ML	B-CLL	Hepatosplenic T-/NK cell ML
No. of cases	29	10	3	13	12
Median age (y)	64.6	64	76	64	54
Sex (M/F)	22:7	7:3	1:2	9:4	7:5
Positive HCV antibody	15	1	0	0	1
Positive HBsAg	6	2	0	1	0
CH/LC/HCC	17:3:1	3:0:0	0	1:0:0	1:0:0
Clinical stage					
I, II	22	4	0	0	0
III, IV	7	6	3	13	12
Splenectomy	25	9	3	0	0
Mean weight of spleen (g)	560	1610	1980	NT	560 at autopsy
Macroscopic feature					
Nodular tumors	29	0	0	0	0
Diffuse infiltration	0	10	3	13	12

Abbreviations. M, male; F, female; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; NT, not tested.

3. Results

3.1. Clinical findings, laboratory data, treatment, and outcome

Clinicopathologically, we classified 29 cases of DLBCL, 10 of SMZL, 3 of CD5-positive mantle cell lymphoma, 1 of hairy cell leukemia, 13 of B-CLL, and 12 of HST/NKL. The clinical data of the 5 ML groups, except for the hairy cell leukemia case, are detailed in Table 2.

3.2. Splenic DLBCL

The median age at diagnosis was 64.6 years (range, 37-83 years). Fifteen (51.7%) splenic DLBCL cases showed serum HCV antibodies, and this prevalence was significantly ($P < .01$) higher than the 5 (9.3%) of 54 nodal DLBCL and 23 (5.2%) of 445 colon cancer cases. Another 6 (20.7%) cases had HBsAg and/or HBeAg, and this prevalence was also significantly ($P < .01$) higher than the 1 (1.9%) of 54 nodal DLBCL cases and 7 (1.6%) of 445 colon cancer cases. Three of the examined HCV-positive cases showed the 1b genotype in their sera and another case was 2b. No type II CG was detected in the 4 examined cases. Twelve cases had a history of chronic hepatitis and 3 had liver cirrhosis, whereas the remaining 6 were healthy carriers. By computed tomography, splenomegaly and large nodular tumors were detected in all the cases. Twenty-five cases received a splenectomy (Fig. 1A), and the mean weight of the removed spleen in these cases was 560 g. In another 4 cases, histological diagnosis was performed using the involved lymph nodes and the liver. Twenty-two cases were classified as stage I or II. Eight cases received a splenectomy, and no further treatments were performed. Combination chemotherapy was performed in 8 cases, and splenic radiation and combination chemotherapy were performed in 7. Six cases were not followed. Eighteen cases showed complete and partial remission from 8 to 84 months. Another 5 cases died, and among these, 4 died of liver dysfunction due to cirrhosis or hepatocellular carcinoma. The 5-year survival of HCV-positive cases was

Table 3 Immunohistological findings and EBV infection of ML

	Diffuse large B-cell	Marginal zone	Mantle cell ML	B-CLL	Hepatosplenic T-/NK cell ML
No. of cases	29	10	3	13	12
CD5	0	0	3	10/12	NT
CD10	6	0	0	0	0
CD20	29	8	3	13	0
Bcl1	0	0	2	0/12	NT
Bcl2	11/24	5	2	12/12	NT
Bcl6	15/24	0	0	0/12	NT
CD25	9/24	0	1/3	7/12	NT
CD3	0	0	0	0	8
TIA1	NT	NT	NT	NT	9/11
CD56	NT	NT	NT	NT	5
HHV-8 LNA	0/24	0/6	0	NT	NT
EBERs (ISH)	1/24	0/6	0	0	5/10

NOTE. Fractions are positive cases/examined cases. Abbreviation: ISH, in situ hybridization.

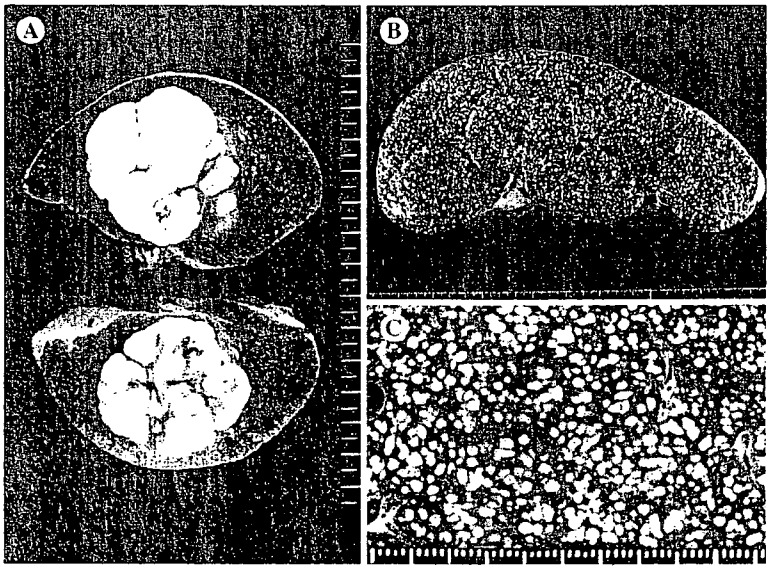

Fig. 1 A, Macroscopic findings of splenic DLBCL with HCV infection. A large lobulated tumor is detected in the spleen. B and C, Macroscopic findings of SMZL without HCV infection. Many small miliary nodules are detected.