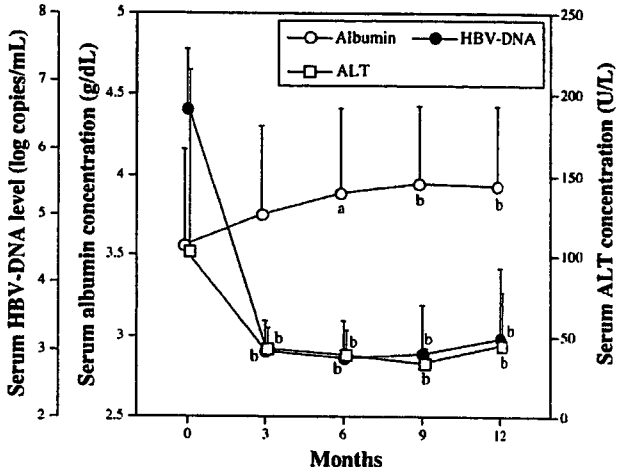

Figure 1
Time course of albumin, HBV-DNA, and ALT levels in lamivudine treatment. The average serum levels of albumin (closed circles), HBV-DNA (open squares), and ALT (open circles) at 3-month intervals from the start of lamivudine therapy are plotted. Soon after the start of treatment, serum albumin levels increased rapidly and simultaneously with a decrease in HBV-DNA and serum ALT levels. The data represent mean + SD (a, b; $p < 0.05$ and $p < 0.01$ vs. 0 month, respectively).

ysis, only HBV-DNA load correlated significantly with Δ-albumin (t = 2.66, $r^2 = 0.120089$, $p = 0.0103$), whereas age, sex, HBeAg, ALT, bilirubin, platelet count, and Child-Pugh classification did not (Table 1).

Although we found no correlation between Δ-albumin and pretreatment serum ALT levels for the entire patient population, the possibility remained that breakthrough hepatitis or continuous elevation of ALT might interfere with Δ-albumin. Indeed, two patients showed breakthrough hepatitis, where ALT levels increased to over 100 U/L, and 20 patients still showed abnormally high ALT (> 35 U/L) at 12 months after treatment. We next evaluated the correlation between Δ-albumin and pretreatment serum ALT levels among the 32 patients in whom serum ALT levels were normalized (< 35 U/L) at 12 months after the start of therapy. As shown in Figure 2A, there was no significant correlation between Δ-albumin and pretreatment serum ALT levels in this subgroup of patients ($r = 0.083$, $p = 0.64$). We also evaluated the correlation between Δ-albumin and reduction in ALT levels at month 12 after starting treatment (Δ-ALT) in this group, but there was still no significant correlation between Δ-albumin and Δ-ALT ($r = 0.0685$, $p = 0.67$) (Figure 2B).

Furthermore, we evaluated the correlation between Δ-albumin and serum HBV-DNA levels before treatment

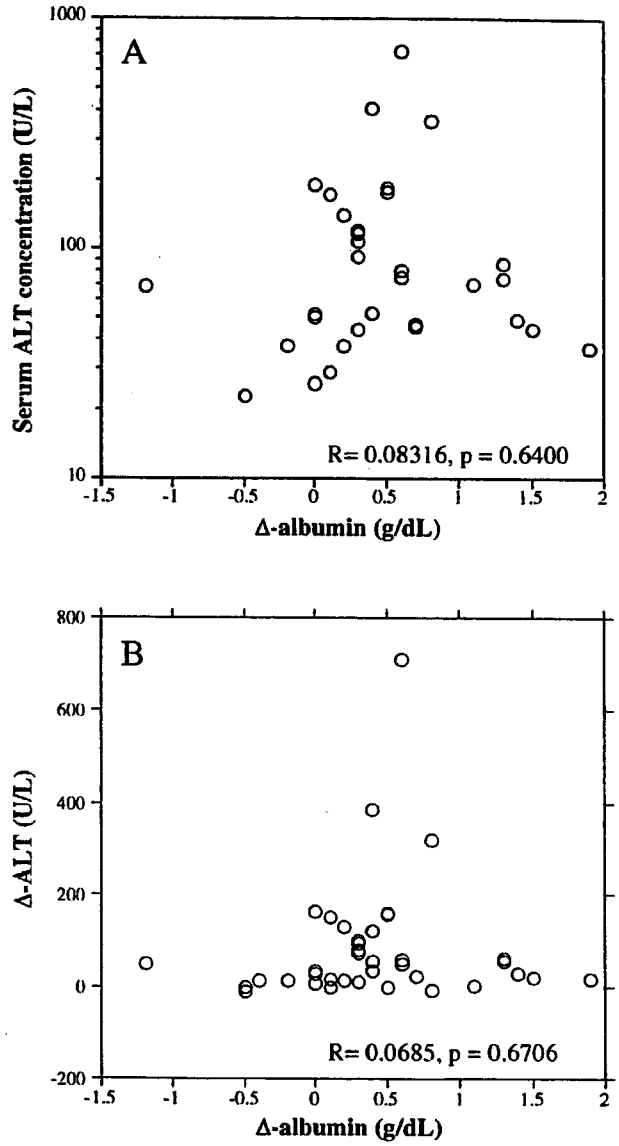

Figure 2
Correlation between ALT levels before treatment and Δ-albumin (A), and Δ-ALT and Δ-albumin (B). In patients whose serum ALT levels were normalized at 12 months after treatment, there was no significant correlation Δ-albumin and pretreatment serum ALT levels (A). There was also no significant correlation Δ-albumin and Δ-ALT (B).

among the 41 patients in whom serum HBV-DNA levels were undetectable at 12 months post-treatment. In this analysis, we found a significant correlation between Δ-albumin and the serum levels of HBV-DNA before the start of therapy ($r = 0.42459$, $p < 0.0001$) (Figure 3A). We also evaluated the correlation between Δ-albumin and reduction in HBV-DNA levels at month 12 after starting

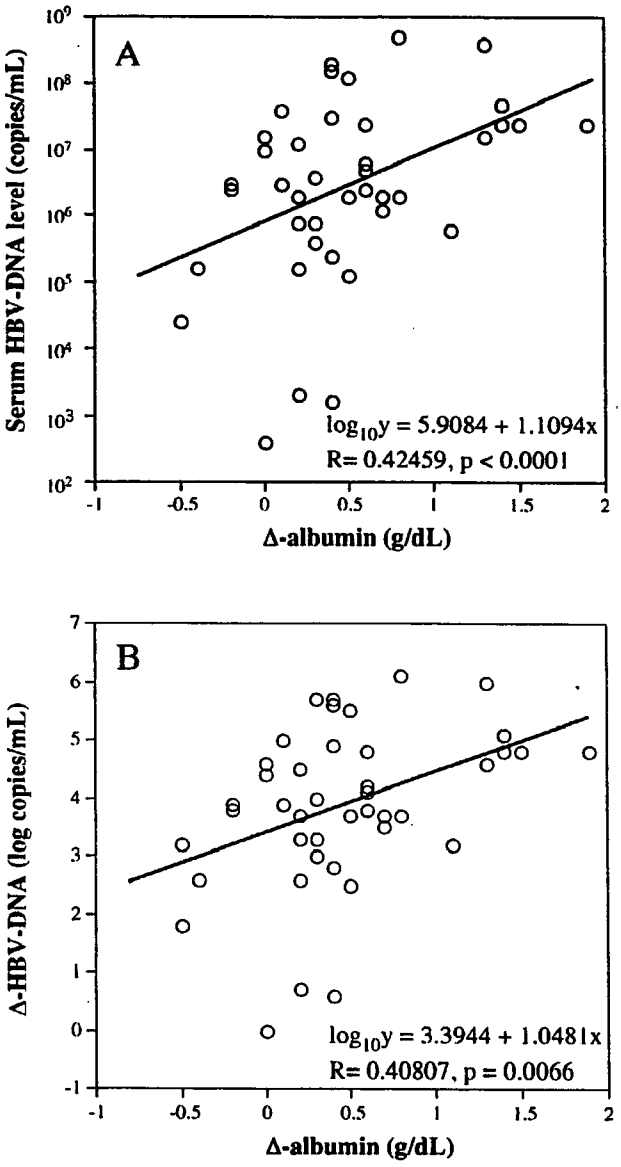

Figure 3
Correlation between HBV-DNA levels before treatment and Δ-albumin (A), and Δ-HBV-DNA and Δ-albumin (B). Patients whose serum HBV-DNA was undetectable at 12 months after treatment, there was a significant correlation between Δ-albumin and both pretreatment serum HBV-DNA levels (A) and Δ-HBV-DNA (B).

treatment (Δ-HBV-DNA) in this group, and we again found that Δ-albumin significantly correlated with Δ-HBV-DNA ($r = 0.40807$, $p = 0.0066$) (Figure 3B).

Discussion

This study demonstrated the followings: 1) HBV-DNA, but not ALT levels, before lamivudine treatment was associated with increased serum albumin levels at 12 months after treatment (Δ-albumin); 2) Even among those patients who showed cessation of hepatitis following treatment, there was no correlation between either pretreatment ALT levels or Δ-ALT and Δ-albumin; 3) In contrast, in the analysis of subjects with undetectable HBV-DNA levels after treatment, there was significant correlation between both pretreatment HBV-DNA levels and Δ-HBV-DNA and Δ-albumin. Taken together, these results suggest that the improvement of hypoalbuminemia following lamivudine treatment is attributable to a reduction of HBV replication, but not to cessation of hepatitis.

We do not deny the idea that cessation of hepatitis, which is represented by lowering of serum ALT levels, contributed to and increase of serum albumin levels. In true, we think that replicative HBV and inflammation are closely related; however, in our study, HBV reduction statistically showed more effect improving serum albumin levels than decreasing the inflammation marker ALT. This may happen perhaps because, in cirrhotic patients, fibrosis is the main pathological change (compared with inflammation), and the correlation between, on the one hand, serum albumin or HBV-DNA levels and, on the other hand, ALT levels was in some degree weakened as the cirrhotic change proceed. Therefore, in cirrhotic patients, Δ-ALT is within a narrower range and ALT levels cannot influence albumin levels significantly.

How does lowering of HBV load induces the increase of albumin levels in an inflammation-independent manner? Hui et al. [7] recently showed that emergence of phenotypic resistance of HBV-DNA was associated with a rapid decline in serum albumin levels following prolonged lamivudine treatment, although they did not report whether a correlation existed between serum ALT levels and serum HBV loads. In a series of studies in woodchucks and Hep G2 cells, Kosovsky et al. demonstrated that HBV replication inversely correlated with cell proliferation and DNA synthesis by hepatocytes [21-23]. Yang et al. has analyzed gene expression profiles of HepG2 cells with or without HBV [24]. However, whether HBV replication directly influences the ability of infected hepatocytes to synthesize protein is still unclear and further studies are needed.

Our results indicate that increased serum albumin levels should be expected in cirrhotic patients following lamivudine treatment, and that this occurs independently of serum ALT levels and Child-Pugh's score before treatment, as shown by the lack of a correlation between those variables and Δ-albumin. Previous studies of lamivudine treatment for liver cirrhosis showed that fatalities occur because of acute liver failure after discontinuation of lamivudine [25,26] or emergence of lamivudine-resistance mutants [27,28]. Recent reports, however, indicate that

Table 2: Correlations between Δ-albumin and basic variables before treatment

	t	R^2	P-value
Age	-0.14	0.000398	0.8873
ALT	0.67	0.008536	0.5064
Bilirubin	-0.04	0.000036	0.9659
Platelet	-0.87	0.014279	0.3894
HBV-DNA	2.66	0.120089	0.0103
HBeAg (+/-)	-	-	0.6201
Sex (male/female)	-	-	0.4251
Child-Pugh's classification	-	-	0.0968

prolonged use of lamivudine for cirrhotic patients is safe and effective [5,29,30]. Furthermore, since adefovir is effective for treating resistant mutants [31-33], lamivudine therapy should be encouraged. Hypoalbuminemia, which causes ascites, edema, and hydrothorax, lowers the quality of life of cirrhotic patients [34,35]. High viral load of HBV is associated with higher mortality and morbidity in cirrhotic patients in consequence of high occurrence or recurrence rate of HCC [36,37]. Lamivudine is effective for preventing or delaying occurrence of liver failure and HCC through lowering HBV, and therefore can be a first choice drug for patients with high HBV levels regardless of serum ALT levels.

Methods

Patients

A total of 54 cirrhotic patients with HBV infection were evaluated, including 38 males and 16 females, ranging in age from 28 to 71 years (mean 52.6 years) (Table 1). Informed consent was obtained from each patient prior to their entering the study. Liver cirrhosis was diagnosed based on liver biopsy (n = 11), laboratory data, ultrasonography, and/or computed tomography. Patients were classified as Child-Pugh class A, B and C (35, 9, and 10 patients, respectively). 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. HBe-Ag was positive in 29 patients and negative in 25 patients. Patients with fatty liver, viral hepatitis C, a history of alcohol abuse, or autoimmune disorders such as autoimmune hepatitis and primary biliary cirrhosis were excluded. None of the patients had a prior history of treatment for hepatocellular carcinoma.

Patients had been treated with lamivudine (100 mg, once a day) without interruption for more than twelve months at Kyushu University Hospital and its affiliated hospitals. Basic laboratory data, such as platelet counts, serum ALT levels, bilirubin, albumin, serum HBV-DNA load (Roche Monitor kit: Roche Diagnostics) and HBe-Ag were determined at least every 3 months.

Statistical analysis

Data are expressed as mean ± SD, and statistical comparisons were performed using chi-squared test for categorical data and one-way ANOVA for numeric data. In cases where the serum HBV-DNA load was less than 2.6 log copies/mL, it was entered as 2.6 log copies/mL. For the analysis of correlations between two continuous variables, a simple regression model was used. For the analysis of discontinuous variables, such as sex and HBe-Ag, statistical differences were confirmed using Mann-Whitney U test or Kruskal-Wallis test.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

MN and ME participated in the experimental design and writing of the manuscript. JH participated in the experimental design. KK performed most of the analysis. YT, EK, JS, AM, TM, NF, HN, HS, KT, KA, and SS collected and supplied the clinical data of patients.

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Original Paper

Fertile Females with Nonalcoholic Fatty Liver Disease (NAFLD) have Higher Levels of ALT than Postmenopausal Females: Implications for the Influence of Fertility on NAFLD

Kazuhiro Kotoh, Makoto Nakamuta, Marie Fukushima, Shusuke Morizono
Munechika Enjoji, Hajime Nawata
Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences
Kyushu University, Fukuoka, Japan
Corresponding Author: Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-5282, Japan
Tel: +81 92 642 5282, Fax: +81 92 642 5287, E-mail: nakamuta@intmed3.med.kyushu-u.ac.jp

KEY WORDS:
Nonalcoholic fatty liver disease (NAFLD); Nonalcoholic steatohepatitis (NASH); Insulin resistance; Sex steroids; Estrogen; Fertility; Postmenopause

ABBREVIATIONS:
Nonalcoholic Steatohepatitis (NASH); Nonalcoholic Fatty Liver Disease (NAFLD); Cytochrome P450 2E1 (CYP2E1); Body Mass Index (BMI); C-peptide (CPR); Fasting Blood Sugar (FBS)

ABSTRACT

Background/Aims: Insulin resistance recently has been reported to play a major role in nonalcoholic fatty liver disease (NAFLD). We evaluated the influence of fertility on fatty liver injury in fertile and postmenopausal women with insulin resistance.

Methodology: We investigated 152 patients with noninsulin-dependent diabetes mellitus without insulin treatment; 46 males, 52 fertile women and 54 postmenopausal women. All had liver damage and/or steatosis recognized by ultrasonography. We measured the fasting serum levels of C-peptide and insulin, as markers of insulin resistance, and the serum levels of ALT. The severity of liver steatosis was judged by ultrasonography.

Results: Fertile females had significantly higher levels of ALT and demonstrated a more significant correlation between serum levels of ALT and C-peptide or insulin than did the postmenopausal females or males. Fertile females with moderate to severe steatosis had significantly higher levels of ALT than those with mild or no steatosis, although such a significant difference was not found in postmenopausal females or males.

Conclusions: We demonstrate that fertility is an important factor in fatty liver damage of NAFLD with insulin resistance, suggesting that estrogen may exacerbate nonalcoholic steatohepatitis.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a worldwide disorder with the potential for progression to cirrhosis, and is generally associated with obesity, diabetes, hypertension and hyperlipidemia (1,2). The progression from simple fatty liver to more severe forms of NAFLD is known as nonalcoholic steatohepatitis (NASH), the histological findings of which are very similar to those of alcoholic liver injury (3). It has been determined that the patients with NAFLD or NASH have insulin resistance and hyperinsulinemia (3,4). Increased serum insulin induces the lipolysis of adipose tissue and elevates the serum levels of free fatty acid (FFA) (3). Therefore, many investigators accepted the hypothesis that the liver injury is caused by the oxidative stress, which usually develops through the mitochondrial oxidation (β-oxidation) of excessive FFA. Unexpectedly, it was revealed recently that β-oxidation and the mitochondrial respiratory chain are impaired (5,6), although whether mitochondrial impairment is a result of enhanced β-oxidation (excess radical production) is not clear. In contrast, several reports have shown that hepatic cytochrome P450 2E1 (CYP2E1) activity was significantly enhanced in patients with NASH, as well as with alcoholic liver disease, and this enhancement also may cause production of cytotoxic radicals (3,7,8). However, it is also the case that many people with both fatty liver and hyperinsulinemia maintain normal liver function (9). This discrepancy suggests that other additional factors can influence the progression of NAFLD.

In the process of searching for potential factors, we noted the uneven gender distribution among patients with NASH (10,11). Most earlier reports showed a high prevalence of female in NASH, indicating that female sex steroids may promote NASH. If estrogen is a key factor for NAFLD or NASH progression, liver injury in fertile women should be greater than in postmenopausal women, although estrogen is well known as an anti-oxidant (12). In this study, to clarify the influence of estrogen on NAFLD, we compared the de-

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gree of the liver injury in fertile and postmenopausal patients. In this study, we investigated the patients with diabetes mellitus complicated with NAFLD and aimed to clarify the influence of gender or menopausal status on the degree of liver injury.

METHODOLOGY

Patients and Methods

Between 1998 and 2003, 282 patients with diabetes mellitus (160 males and 132 females) were hospitalized in our department, Kyushu University Hospital. Patients with insulin dependent diabetes mellitus (4 males and 6 females), habitual alcohol drinkers (105 males and 8 females), patients with chronic viral hepatitis and patients with complications of cancer were excluded. Three pregnant women and five fertile patients with abnormalities of ovulation also were excluded. The remaining 46 males and 106 females (52 fertile and 54 postmenopausal women) were investigated in this study. The body mass index (BMI) was calculated using the height and the weight on the day of admission. The serum levels insulin or C-peptide (CPR), or both, were measured after overnight fasting within five days of admission. On the same day, the serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), alkaline phosphatase (ALP), fasting blood sugar (FBS), total cholesterol, triglyceride, albumin and HbA1c also were measured.

Although abdominal ultrasonography was performed for all patients, the results of five patients were excluded from the analysis because they underwent the examination over seven days after admission. The severity of fatty liver on ultrasonography was judged by two expert examiners as follows: When the echo level of the liver was elevated compared with that of the kidney cortex, the patient was diagnosed as having fatty liver. If the wall of the portal vein at the umbilical portion provided a clear image, the disease was classified as mild. When the wall of portal vein was partially or wholly undetectable at the same position, according to the echo level elevation of liver parenchyma, the disease was classified as moderate or severe fatty liver, respectively.

Statistical Analysis

Data of the basal characteristics of the patients are presented as mean ± standard deviation (SD). Student's unpaired *t* test was used to compare the pairs of subgroups. Sheffe's F test following the analysis of variance (ANOVA) was used to analyze the influence of the severity of steatosis on liver injury. Stepwise multiple linear regression analysis was used to investigate independent variables that might influence the serum levels of ALT. Independent variables tested included BMI, CPR, insulin, FBS, cholesterol, triglyceride and HbA1c. Stepwise multiple linear regression analysis was performed for each subgroup, males, females, fertile women and postmenopausal women.

RESULTS

When we compared the laboratory data concerning liver function and glucose tolerance between male and female patients, there was no significant difference except for the serum levels of albumin (**Table 1**). The comparisons of serum levels of cholesterol and triglyceride, and BMI, also showed no significant differences between the genders. Upon dividing the female patients into fertile and postmenopausal groups, the serum levels of ALT in fertile females were significantly higher than those in postmenopausal females, while the FBS was significantly lower (**Table 2**). Although the difference was not significant, the results

TABLE 1 Characteristics of the Patients

	Male	Female	All
Number	46	106	152
Age	54.5±14.7	49.3±14.4	50.9±14.6
BMI (kg/m²)	25.1±6.6	26.5±6.5	26.1±6.5
AST (U/L)	20.9±9.7	25.0±16.2	23.8±14.7
ALT (U/L)	26.4±23.8	31.4±27.3	29.9±26.3
γ-GTP (U/L)	37.2±19.6	37.5±31.9	37.4±28.7
ALP (U/L)	242.3±66.5	240.0±91.8	240.4±84.8
Cholesterol (mg/dL)	205.4±46.0	213.7±40.7	211.3±42.3
Triglyceride (mg/dL)	183.4±125.0	162.1±100.8	168.6±108.7
FBS (mg/dL)	184.4±75.6	167.7±66.6	172.7±69.6
Albumin (g/dL)	3.9±0.6	4.1±0.4*	4.1±0.5
HbA1c (%)	9.2±2.2	8.6±2.2	8.8±2.2
CPR (ng/mL)	1.9±1.3	2.2±1.3	2.1±1.3
Insulin (μU/mL)	8.3±8.8	11.3±8.5	10.4±8.6
Steatosis on US			
Normal	19	18	37
Mild	13	43	57
Moderate	9	31	40
Severe	3	10	13

*$p<0.05$ *vs.* male.

TABLE 2 Characteristics of the Patients, Comparing Fertile and Postmenopausal Women

	Fertile women	Postmenopausal women
Number	52	54
Age	38.3±10.8	60.0±7.9**
BMI (kg/m²)	26.5±6.9	26.6±6.1
AST (U/L)	28.1±21.3	22.1±8.0
ALT (U/L)	37.4±34.6	25.7±15.9*
γ-GTP (U/L)	40.9±37.3	34.3±25.5
ALP (U/L)	221.1±82.0	257.7±97.9*
Cholesterol (mg/dL)	214.7±47.8	212.7±33.1
Triglyceride (mg/dL)	149.0±75.1	174.3±119.2
FBS (mg/dL)	152.7±67.2	182.1±63.3*
Albumin (g/dL)	4.1±0.4	4.2±0.4
HbA1c (%)	8.3±2.7	8.9±1.7
CPR (ng/mL)	2.1±1.3	2.4±1.3
Insulin (μU/mL)	10.5±8.5	12.1±8.4
Steatosis on US		
Normal	10	8
Mild	21	22
Moderate	13	18
Severe	5	5

*$p<0.05$ *vs.* fertile women, **$p<0.001$ *vs.* fertile women.

TABLE 3 Stepwise Multiple Linear Regression Model for Independent Predictors for Serum Levels of ALT

Category	Variables	SE	β	*p* value
Male	BMI	0.678	0.452	0.0002
	Insulin	0.438	0.309	
Female	CPR	2.084	0.550	<0.0001
Fertile women	CPR	3.710	0.682	<0.0001
Postmenopausal women	CPR	1.476	0.556	0.0001

β: standardized coefficient.

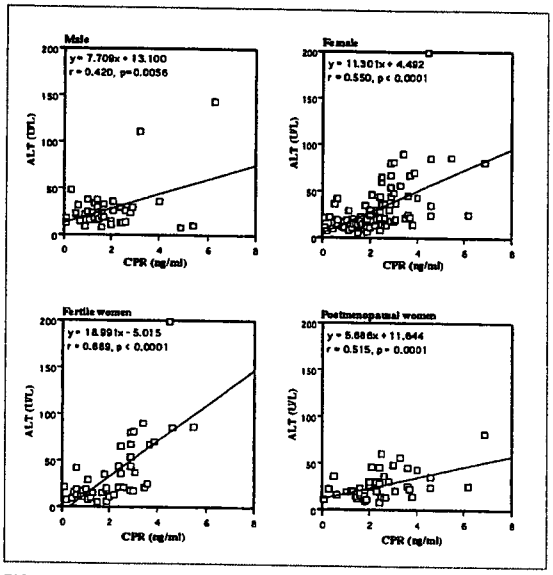

FIGURE 1 The correlation between serum levels of ALT and CPR was examined separately in males and females (upper two panels). When female patients were divided into fertile and postmenopausal groups, the former showed both a stronger degree of correlation and much higher levels of ALT than the latter (lower two panels). The fertile females had about threefold higher levels of ALT compared to the menopausal females with the same level of insulin.

of HbA1c, CPR and insulin also showed that the postmenopausal group had poorer glucose tolerance, which indicated that the postmenopausal females had more severe insulin resistance. On the other hand, BMI and the severity of fatty liver on ultrasonography were similar between the two groups. In order to confirm that this difference was not merely related to age, the male patients were divided into two groups according to age and were compared for the same factors, which revealed that there was no difference between the younger and the older males (data not shown).

Many investigators have reported that insulin resistance is an important factor in the progression of NASH, and it is accepted that the serum levels of CPR and insulin of fasting patients correlate well with the degree of insulin resistance. In order to confirm that the influence of insulin resistance is similar regardless of gender or menopause, stepwise multiple regression analysis was performed for males, females, and fertile and postmenopausal women (**Table 3**).

For all females, and for fertile and postmenopausal women, CPR was evaluated as a variable that was significantly correlated with the serum levels of ALT, whereas BMI and insulin were evaluated in males. By further analysis of the correlation between the serum levels of ALT and those of CPR or insulin, it was revealed that the degree of the correlation varied among the subgroups. As shown in **Figure 1**, both male and female patients showed a weak but significant correlation between the serum levels of CPR and those of ALT, and the correlation curves were similar. In dividing females into fertile and postmenopausal groups, however, their correlation curves were apparently different. Furthermore, the correlation, particularly in the fertile group, was stronger than that of all females or males. These findings indicated that a fertile patient would have about a threefold higher ALT level compared to a postmenopausal patient with the same CPR level. In addition, the correlation curve of postmenopausal women was almost equal to that of all males. We obtained similar results from analysis of the correlation between the serum levels of insulin and those of ALT (**Figure 2**).

We also analyzed the correlation between the serum levels of ALT and the severity of steatosis, another factor known to be related to NASH progression. In males and postmenopausal women, although the average level of ALT increased according to the progression of fatty liver, the difference was not significant (**Figure 3**). In the fertile group, however, the patients with moderate to severe fatty liver showed significantly higher levels of ALT than those without steatosis or with mild fatty liver.

DISCUSSION

Recent studies revealed that other factors, in addition to steatosis, are involved in the progression of NAFLD or NASH; such factors are so-called "second hit" (3) because many people with fatty liver and hyperinsulinemia maintain normal liver function (9). As shown in our study, the severity of fatty liver did not correlate significantly with the serum levels of ALT in males and postmenopausal women. Previous reports have suggested several candidates as "second hit" factors, such as insulin resistance, cytokines, oxidative stress, and so on (3,13). Among these, it has been proven that most patients with NAFLD or NASH have insulin resistance (1,3,14), however, the combination of steatosis and insulin resistance is not sufficient to explain the mechanism for all patients. Therefore, it has been suggested that oxidative stress and subsequent lipid peroxidation may be the ultimate factors which cause inflammation and fibrosis in the liver. Nevertheless, this final step for progression of NHLD or NASH has not yet been proven directly.

Before embarking on this study we formed the hypothesis that the liver damage might be moderate in fertile patients because of the well known anti-oxidative properties of estrogen. There is a large body of ev-

idence that oxidative stress is implicated in chronic liver disease and serves as a link between hepatic injury and fibrosis. It has been reported that estradiol inhibits liver inflammation and fibrosis caused by CCl_4 or dimethylnitrosamine in animal models (15,16). Shimizu *et al.* also suggested that the rapid progression to cirrhosis in men and postmenopausal women with hepatitis C might be due to the low level of estradiol (17). In addition to its anti-oxidant effect, it has been reported that estrogen reduces the expression of cell adhesion molecules *in vivo* and *in vitro*, which indicates that estrogen might have an anti-inflammatory role (18,19).

In our study, both fertile and postmenopausal women showed good correlations between the serum levels of ALT and CPR or insulin, which indicates that hyperinsulinemia or insulin resistance is a key factor in the progression of NAFLD. We also found that HOMA index (FBS x insulin /405) also had weaker correlation to the serum level of ALT than CPR or insulin (data not shown), although HOMA index is not a good marker of insulin resistance when the level of FBS is over 170mg/dL. Although our findings are in agreement with previous reports (3), our results are contrary to our hypothesis that the degree of liver injury would be moderate in fertile females with higher estrogen levels. Fertile females clearly showed a stronger correlation between ALT levels and CPR levels than did postmenopausal females, indicating that estrogen is possibly an aggravating factor.

If estrogen is one of the 'second hit' factors of NAFLD or NASH, how might it affect the progression of the disease? Several lines of evidence have been reported that estrogen suppresses lipid oxidation in the liver and increases the activity of CYP2E1. Lipid oxidation is reduced in pregnant women compared with healthy non-pregnant females, who in turn have lower lipid oxidation than postmenopausal females (20). In addition, serum estradiol correlated negatively with lipid oxidation (20). Furthermore, oral estrogen replacement in postmenopausal females suppresses hepatic lipid oxidation more significantly than transdermal replacement, because of a first pass effect (21). With regards to CYP2E1, its activity in female mice is higher than in male mice, and the activity in male mice was significantly increased by treatment with estradiol (22). The situation of both suppression of lipid oxidation and enhancement of CYP2E1 by estrogen is very similar to that observed in NASH, as well as alcoholic liver disease. Toremifene, an estrogen receptor antagonist, recently has been reported to alleviate ethanol induction of CYP2E1, resulting in protection of female rats from alcoholic liver injury (23). These evidences suggest that estrogen possibly exacerbates liver damage in NAFLD or NASH despite its anti-oxidative properties, and we now are evaluating the effect of withdrawing estrogen by ovariectomy in NASH model mice.

Because the menopause clearly means a dramatic withdrawal of estrogen, indicating that androgenic

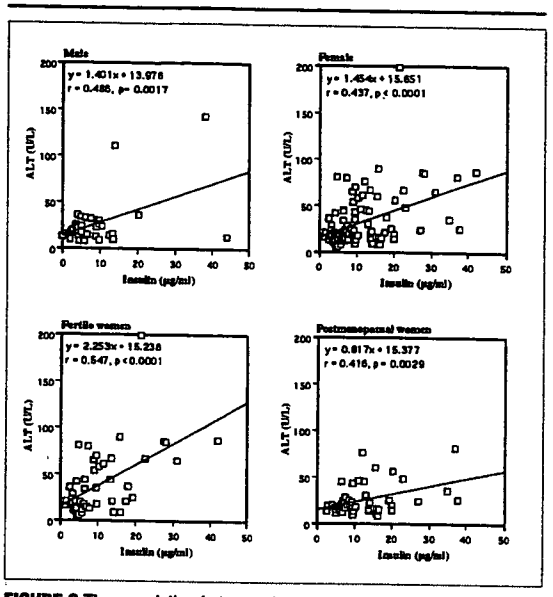

FIGURE 2 The correlation between the serum levels of ALT and insulin was examined separately in males and females (upper two panels). The correlations were significant but weaker in females when compared with the correlation between the serum levels ALT and CPR. Comparing fertile and postmenopausal women, similar results were obtained to those in Figure 1 (lower two panels).

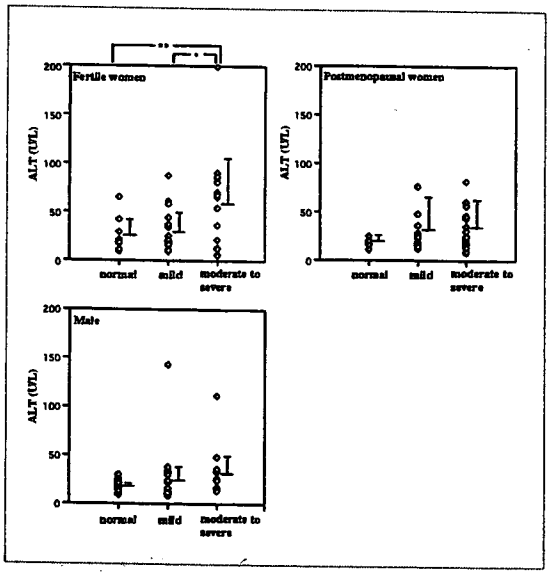

FIGURE 3 The influence of the severity of fatty liver on the serum levels of ALT was examined. In males and postmenopausal women, the average ALT levels tended to be high according to the progression of steatosis in all subgroups, but the difference was not significant. In fertile women, however, the patients with moderate to severe fatty liver showed significantly higher levels of ALT than those without steatosis or with mild fatty liver.

effects become relatively increased, we have to pay attention to androgenic roles in NFALD or NASH. It has been reported that male rats show higher levels of hepatic peroxisome proliferator-activated receptor (PPAR) alpha protein, the activation of which simulates oxidation, and that this difference is abol-

ished by gonadectomy of male rats (24). In our analysis of male patients, their ALT-CPR correlation curve was similar to that of postmenopausal women. However, the correlation was weak, and was not reliable because the population with high insulin resistance was very small. This low population was mainly due to the exclusion of a large number of patients who habitually drank alcohol. A study involving a larger number of patients would be required to confirm the influence of androgens. In conclusion, our results strongly indicate that sex steroids are key factors of progression of NAFLD and NASH. If estrogen is an aggravating factor, our findings might lead to understanding of two unresolved clinical problems, the mechanism of acute fatty liver in pregnancy and the rapid progression of alcoholic hepatitis in females. Further studies focusing on the roles of sex steroids in NFALD and NASH are needed, both clinical studies and animal experiments using their models.

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BASIC STUDIES

Decreased portal flow volume increases the area of necrosis caused by radio frequency ablation in pigs

Tsuyoshi Yoshimoto, Kazuhiro Kotoh, Yuki Horikawa, Motoyuki Kohjima, Shusuke Morizono, Shinsaku Yamashita, Munechika Enjoji and Makoto Nakamuta

Department of Medicine and Bioregulatory Science, Graduate School of Medical Science, Kyushu University, Fukuoka, Japan

Keywords
complication – hepatocellular carcinoma – over-ablation – RFA

Correspondence
Kazuhiro Kotoh, Department of Medicine and Bioregulatory Science, Graduate School of Medical Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
Tel: +81 92 642 5282
Fax: +81 92 642 5287
e-mail: kotoh-k@intmed3.med.kyushu-u.ac.jp

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Abstract

Background/aims: Although radio frequency ablation (RFA) has been widely accepted as an effective treatment for hepatocellular carcinoma (HCC), severe complications are not uncommon. Major complications seem to occur as a result of over-ablation beyond the intended area. As most patients with HCC have underlying cirrhosis, we speculated that decreased portal flow might cause the necrosis associated with RFA. To confirm this hypothesis, we examined the area of necrosis resulting from RFA under varying conditions of portal flow in a porcine model. *Methods:* RFA was performed using ultrasonographic guidance in anesthetized pigs. During the RFA procedure, portal flow was regulated by a balloon catheter, which was set in a portal trunk. The necrosis area was measured after sacrifice and was compared with the hyperechoic area that appeared during ablation. In another session, RFA was performed close to the hepatic vein and endothelial damage was examined. *Results:* The necrosis area caused by RFA was significantly larger when the portal flow volume was decreased by 50% or more. The hyperechoic lesion was always larger than the area of pathological necrosis regardless of portal flow volume. Under conditions of decreased portal flow, the vessel endothelium near the ablated area was more readily damaged. *Conclusion:* Decreased portal flow volume resulted in enlargement of the area of necrosis caused by RFA. Our results indicate that over-ablation could easily occur in patients with advanced cirrhosis, and that this could lead to major complications. Ultrasonographic guidance may be helpful for avoiding over-ablation.

The efficacy of radio frequency ablation (RFA) for the treatment of hepatocellular carcinoma (HCC) has been widely recognized (1–5). Recent studies comparing RFA with earlier ablation methods such as ethanol injection therapy and microwave coagulation therapy showed that RFA resulted in wider and more complete necrosis (3, 6, 7). However, several studies have also shown that RFA can be associated with serious complications such as gastrointestinal perforation, hepatic abscess, intraperitoneal hemorrhage, portal thrombus, liver infarction, and bile peritonitis (4, 8–12). Despite the prevalence of such complications, the background of patients most likely to experience complications related to RFA has not been investigated.

Most of the reported complications seem to have occurred when the ablated area spread beyond the intended margin, which caused heat damage in vessels, bile ducts, and contiguous viscera. Because the RFA procedure has been generally performed using two-dimensional ultrasonographic imaging, it is conceivable that vessels or viscera located apart from guiding-image plane could be unexpectedly injured. However, not only is it difficult to visualize three-dimensional structure but the actual ablation area achieved with RFA may not always be equal to that which was intended. In our limited experience, we have found that the area of ablation confirmed by computed tomography after RFA was not equal among patients despite treatment with the same protocol and device. To our knowledge, such unevenness in the ablation area among patients has not been widely addressed. However, it is important to determine why unevenness of the ablated area occurs, as it could contribute to complications associated with RFA.

To explain why the ablated area is often uneven, we speculated that the portal flow volume, which is decreased to varying degrees among patients with liver cirrhosis, would influence the ablation size. It is well known that the most of patients with HCC have liver cirrhosis owing to hepatitis B or C virus. It has

previously been shown in a porcine model that RFA during the Pringle maneuver spreads the ablated area owing to a 'heat sink effect' (13, 14). However, most cirrhotic patients have at least some residual portal flow volume (15, 16), while the Pringle maneuver results in complete occlusion of portal flow. In discussing the influence of portal flow volume on RFA in the patients with liver cirrhosis we should confirm the effects under various degree of portal flow volume.

To determine the influence of portal flow volume on RFA in patients with liver cirrhosis we used a porcine model in which the portal flow volume was varied using a balloon catheter set in the portal vein. We compared the histological ablation area and the expected ablation area based on ultrasonographic imaging, as differences between these two parameters may contribute to complications associated with RFA.

Methods

Animals

Five healthy female pigs (58–61 kg) were anaesthetized with ketamine 10 mg/kg, clonidine 5 μg/kg, and atropine 0.02 mg/kg. Anaesthesia was maintained by ventilation with a mixture of oxygen and nitrous oxide. The RFA procedure was performed following midline laparotomy and the animals were subsequently sacrificed.

Regulation of portal flow volume and RFA

In order to regulate the portal flow volume, a balloon catheter (Occlusion Balloon Catheter, OBW/20/8/100, Boston Scientific Japan K.K., Tokyo, Japan) was set in the main trunk of the portal vein and X-ray imaging was used to confirm that the tip of the catheter was located within the main trunk. The portal flow volume was measured at the first right branch of the portal vein by expanding the balloon to various degrees before puncture with the electrode needle.

The electrode needle was a LeVeen™ multipolar array needle (20 mm diameter type; Boston Scientific Corporation, Natick, MA, USA) used in combination with an RF 2000 generator™ (Radio Therapeutics Corporation, Sunnyvale, CA, USA) according to the manufacturers protocol. In short, the tines were fully expanded after the needle was inserted to the target position and RF energy was then applied to the tissue using an initial power setting of 30 W, which was subsequently increased in increments of 10 W/min to a maximum power of 75 W. The power setting was then maintained at 75 W until power 'roll-off' occurred; tissue impedance (an increase in tissue resistance caused by decreased conductivity of electrical current by protein denaturation and loss of intracellular fluids) rose to over 200 ω, at which time the power passively decreased to < 10 W. For the ablations, all electrode needle punctures were performed under ultrasonographic guidance confirming that visible vessels were at least 20 mm away from the expanded electrode.

After ablation, the hyperechoic areas on ultrasonographic images were evaluated. The maximum and minimum dimensions (length and width) of the hyperechoic lesion were determined and the area was calculated as follows:

$$\text{Area of hyperechoic lesion} = ((\text{maximum distance} + \text{minimum distance})/4)^2 \times \pi.$$

We also performed RFA close to the hepatic vein to observe the influence of portal flow volume on blood temperature in the hepatic vein and on endothelial damage. For these procedures, the tip of the electrode was placed 10 mm from hepatic vein and a thin thermometer (Digital Thermometer; SK-250WP, Sato Co., Tokyo, Japan) was set in the vein during the ablation.

Data shown in the figures are expressed as mean ± SD. Dunnett's *post hoc* test was used to analyse differences in the ablated areas from the RFA group and the non-occlusion control group.

Histopathology

To determine the area of necrosis caused by RFA, the ablated lesion was removed *en bloc* and sectioned tangential to the long axis of the probe shaft. For each specimen, the maximum and minimum distances of the region of necrosis area were measured, and the area of necrosis was calculated as follows:

$$\text{Pathological necrosis area} = ((\text{maximum distance} + \text{minimum distance})/4)^2 \times \pi.$$

Portal vein specimens including liver parenchyma were taken from near the ablated area, cut into blocks, and frozen in liquid nitrogen. Cryostat sections (8 μm) were fixed with 4% (w/v)-buffered formaldehyde, rinsed with distilled water, and mounted in glycerol jelly. Sections were incubated with a solution containing the substrate NADH (Sigma Diagnostics, St. Louis, MO, USA) and the indicator nitro-blue tetrazolium (Sigma Diagnostics) for 30 min at room temperature. Cell viability was judged by the detection of reduced nicotinamide adenine dinucleotide diaphorase (NADHd).

Results

When the balloon in the portal vein trunk was expanded, no change of blood pressure, heart rate, or respiration was observed. The RFA procedure and the puncture of the hepatic vein did not affect vital signs.

As shown in Fig. 1A, the pathological necrosis area negatively correlated with the portal flow volume. The areas of necrosis under conditions of 25% occlusion did not differ significantly from the control group (0% occlusion), whereas the area was significantly increased by occlusion of 50% or more. We also examined the effect of RFA just after sacrifice to determine the effect on necrosis area when both portal vein and hepatic artery flow was interrupted. The necrosis area caused by RFA after sacrifice was almost equal to that under conditions of 100% portal vein occlusion.

The hyperechoic area produced by RFA gradually increased as a result of portal vein occlusion and was consistent with pathological findings. We aimed to separately examine the US-image change and the pathological change. After that, we confirmed the correlation between them. (Fig. 1B). The pathological necrosis area was always larger than the hyperechoic area determined by ultrasonographic imaging regardless of the degree of portal vein occlusion (Fig. 1C).

In the series of ablations conducted close to the hepatic vein, the increase of intravenous temperature was significant with 100% portal vein occlusion but not with 50% occlusion (Fig. 2). NADHd staining of hepatic vein specimens revealed massive loss of endothelial cells in all of the samples from the group that received 100% portal vein occlusion. Endothelial cell viability was decreased by expansion of the balloon in the portal vein (Fig. 3).

Discussion

Although the effect of RFA for HCC has been confirmed in past studies, reports of complications were not infrequent. In recent analyses of large numbers of patients with HCC, the rate of major complications was 4.0–5.7% (4, 8, 9, 12). Despite the relatively high frequency of complications, the backgrounds of patients who are most likely to develop complications have rarely been discussed. Recently, Curley et al. (12) prospectively analysed the complications related to RFA for primary and metastatic liver tumors and showed that the prevalence was higher in cirrhotic than in non-cirrhotic patients. However, they also pointed out that there was only a slight difference in the rate of complications between these two groups of patients, and emphasized that the higher frequency of complications in cirrhotic patients was acceptable because most patients with advanced liver cirrhosis would not be candidates for liver resection. Although we consider their results to be merely suggestive, the correlation between the prevalence of complications and the degree of cirrhosis progression should be analysed in order to clarify the importance of cirrhosis as a predictive factor for the occurrence of complications.

On the basis of the earlier studies that showed increased ablated area under Pringle maneuver, it is natural to speculate that decreased portal flow volume might negatively influence the ablation size with RFA. Several prior studies have evaluated portal flow volume in cirrhotic patients using Doppler ultrasound. According to one study, the portal flow volume in Child's A patients was maintained at about 90% of healthy controls, whereas that in Child's B or C patients decreased to about 50–60% (16). Our results

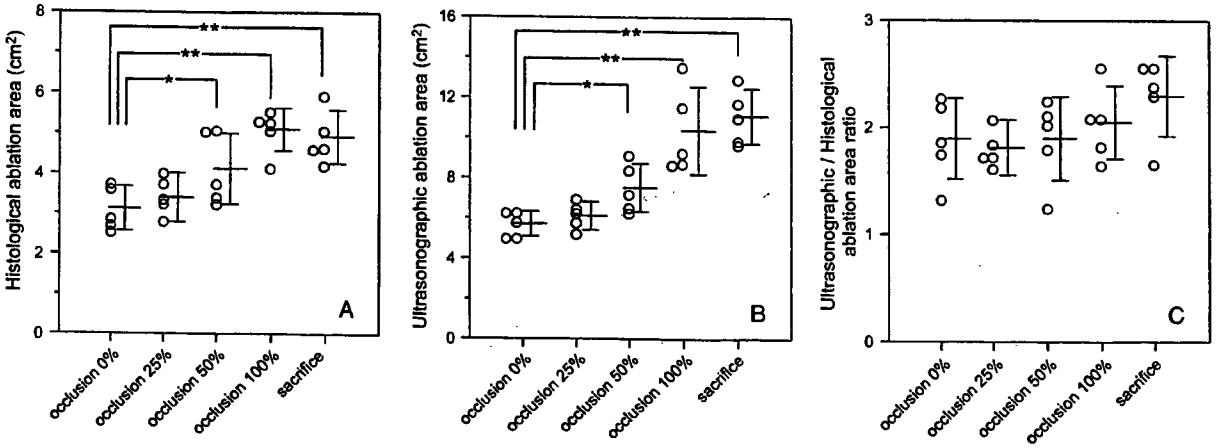

Fig. 1. Both the pathological necrosis area (A) and the hyperechoic area (B) caused by radio frequency ablation (RFA) negatively correlated with portal flow volume. The hyperechoic area was always larger than necrosis area regardless of portal flow volume (C).

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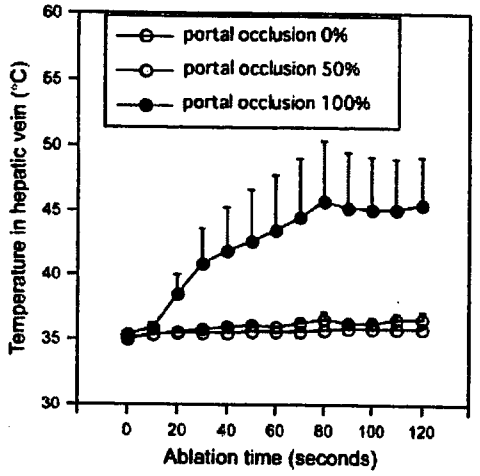

Fig. 2. Intravenous temperature during ablation close to the hepatic vein. The temperature significantly increased as a result of 100% occlusion of portal flow, while temperature did not change significantly with either 50% or 0% occlusion.

revealed that a decrease in portal flow volume of 50% or more caused a significant increase in the ablation size, indicating that unexpected spread of the ablation area might occur with RFA in advanced cirrhotic patients. Furthermore, we also observed an increase of intravenous temperature and endothelial damage associated with ablation close to the hepatic vein under conditions of decreased portal flow volume. Unintentional spread of the ablation area might cause adjacent visceral perforation and bile duct injury, and vessel endothelial damage might lead to thrombosis. Although such complications were commonly reported in prior clinical studies of RFA for liver tumor treatment, we noticed that the incidence of intestinal perforation has decreased in recent reports. We speculate that this decrease may be owing to improved technical skill of the operators who are more proficient in avoiding critical complications. If this assumption is true, then the negative influence of portal flow volume on ablation area could represent a greater risk for immature operators.

In recent studies, the percutaneous ultrasound-guided procedure has been used more often than has RFA with laparoscopy (5, 17, 18). With the ultrasound-guided technique, the hyperechoic area is regarded as the margin of necrosis. However, no prior studies have confirmed whether the hyperechoic image during ablation actually corresponds with histological necrosis. In order to confirm the usefulness of ultrasonographic imaging during RFA, we compared the histological necrosis area after RFA with the hyperechoic area achieved by thermal ablation. Our result

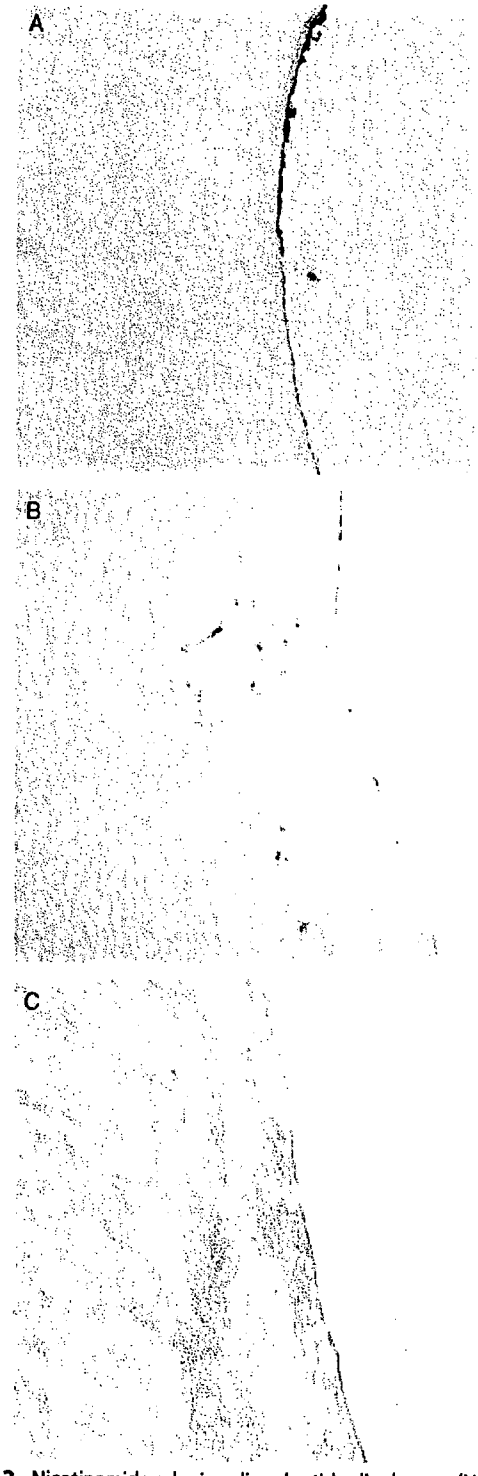

Fig. 3. Nicotinamide adenine dinucleotide diaphorase (NADHd) staining of sections of liver tissue obtained following ablation close to the hepatic vein under conditions of 0% (A), 50% (B), and 100% occlusion (C) of portal flow. The viability of venous endothelial cells declined in proportion to the decrease in portal flow volume.

showed that the area of histological necrosis was always smaller than the ultrasonographic image regardless of the portal flow volume. Hence, major complications can be avoided when the ablation procedure is performed using the spread of the hyperechoic image on ultrasonography as a guide.

Our results indicate that the necrosis area caused by RFA is influenced by portal flow volume. Considering that most of the patients with HCC have underlying cirrhosis, the unevenness of ablation size, even when using the same device and protocol, is likely owing to differences in portal flow volume. When using RFA in cirrhotic patients, especially those with Child's B and C, the resultant area of ablation may be larger than intended, which could lead to severe complications. Careful observation of the hyperechoic image that develops during ablation when using ultrasonographic guidance can be helpful for avoiding major complications related to RFA.

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Specific mutations in enhancer II/core promoter of hepatitis B virus subgenotypes C1/C2 increase the risk of hepatocellular carcinoma

Yasuhito Tanaka[1], Motokazu Mukaide[1,3], Etsuro Orito[2], Man-Fung Yuen[4], Kiyoaki Ito[2], Fuat Kurbanov[1], Fuminaka Sugauchi[2], Yasuhiro Asahina[5], Namiki Izumi[5], Michio Kato[6], Ching-Lung Lai[4], Ryuzo Ueda[2], Masashi Mizokami[1,*]

[1]*Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan*
[2]*Internal Medicine and Molecular Science, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan*
[3]*SRL Inc., Tokyo, 51 Komiya, Hachiouji, Tokyo 192-8535, Japan*
[4]*Department of Medicine, The University of Hong Kong, Queen Mary Hospital, Hong Kong*
[5]*Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan*
[6]*National Hospital Organization Osaka National Hospital, Osaka, Japan*

***Background/Aims*: Hepatitis B virus genotype C (HBV/C) has been classified into two geographically distinct subgenotypes; HBV/C1/Cs (Southeast Asia) and HBV/C2/Ce (East Asia).**

***Methods*: Viral differences in enhancer II/core promoter and precore regions between the subgenotypes and their association with hepatocellular carcinoma (HCC) were assessed in a matched cross-sectional control study of 118 carriers (from Hong Kong) with HBV/C1/Cs (48.0 years, 81% male, 40% HBeAg+, 44% HCC) and 210 HBV/C2/Ce (172 from Japan, 38 from Hong Kong) (50.2 years, 78% male, 30% HBeAg+, 46% HCC).**

***Results*: Univariate analyses showed that mutation V1753 was predictive for HCC among HBeAg-positive-C1/Cs-carriers ($P = 0.0055$), and T1653 among HBeAg-positive-C2/Ce-carriers ($P = 0.018$), and T1653 or V1753 or T1762/A1764 among HBeAg-negative-C2/Ce-carriers ($P < 0.05$). In the multivariate analysis on all HBV/C subjects, independent predictive factors for HCC were subgenotype C2/Ce (odds ratio, 4.21; 95% confidence interval, 1.07–16.23), T1653 (3.64; 1.93–6.86), V1753 (3.07; 1.66–5.65) and T1762/A1764 (2.58; 1.21–5.49) mutations, age (≥50 years), gender (male) and HBeAg (positive).**

***Conclusions*: Our data indicate that T1653 and/or V1753 mutations in addition to T1762/A1764 are differently associated with HCC in context of HBeAg status among HBV/C1/Cs and C2/Ce-carriers. HBV/C subgenotypes have specific mutation patterns, which is probably responsible for increased carcinogenesis of HBV/C2/Ce.**

***Keywords*: Hepatitis B virus; Hepatocellular carcinoma; Subgenotype C; Enhancer II; Core promoter; Precore genome**

1. Introduction

HBV genotypes have a distinct geographical distribution and correlate with severity of liver disease [1,2]. Genotypes B and C are prevalent in Asia, and genotype C causes more serious liver disease than genotype B [3,4]. There are two subtypes (subgenotypes) of genotype B in distinct geographical distributions, designated Ba ("a" standing for Asia) and Bj ("j"

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* Corresponding author. Tel.: +81 52 853 8292; fax: +81 52 842 0021.

E-mail address: mizokami@med.nagoya-cu.ac.jp (M. Mizokami).

Abbreviations: HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; ALT, alanine aminotransferase; ELISA, enzyme-linked immunosorbent assay; RFLP, restriction fragment length polymorphism; RTD-PCR, real-time detection polymerase chain reaction.

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for Japan) provisionally [5], and clinical differences between patients infected with HBV/Ba and HBV/Bj are coming to the fore [6,7]. Recently, a phylogenetic analysis of the pre-S1/pre-S2 genes revealed two major groups within genotype C: one for strains from Southeast Asia including Vietnam, Myanmar, Thailand and Hong Kong (named HBV/C1) and the other for strains from (Far) East Asia including Japan and China (named HBV/C2). This finding was confirmed by phylogenetic analyses based on the complete sequences of 32 HBV/C strains [8], and by recent independent studies in Hong Kong [9] and Japan [10]. The latter papers designated the 2 subgenotypes as HBV/Cs in Southeast Asia and HBV/Ce in the (Far) East Asia that have not only different epidemiological distributions but also different virological findings in precore regions [9,10].

Mutations in the basic core promoter (BCP) region at nucleotides (nt) 1762/1764 (T1762/A1764) and mutation in the precore region at nt 1896 (A1896) are associated with HBe antigen seroconversion (SC) and viral replication. It is noteworthy that the both BCP and precore stop-codon mutations are often found in patients with advanced liver disease such as hepatocellular carcinoma (HCC) [11–14]. Beyond these mutations, the C to T mutation in the upstream regulatory sequence (C1653T) is associated with fulminant hepatitis [15] and located in the alpha box, which is a strong activating element of both enhancer II and core promoter [16]. Takahashi et al. [17,18] reported that C-to-T1653 and T-to-V(not T)1753 mutants were more closely associated with the progression of liver disease from chronic hepatitis to cirrhosis and/or HCC in HBeAg-positive patients, compared with the BCP double mutation. Our recent case-control study supports that the addition of T1653 mutation in enhancer II to the BCP mutation increases the risk of HCC in anti-HBe-positive patients with HBV/C [19].

To evaluate clinical and virological significances between HBV/C1/Cs and HBV/C2/Ce, in the present study, we performed a multi-center cross-sectional matched control study among HBV/C carriers [inactive carriers (IC), chronic hepatitis (CH), HCC] and determined the specific HBV mutations associated with disease progression.

2. Materials and methods

2.1. Serum samples

A total of 328 sera were obtained from chronic HBV/C carriers who visited Nagoya City University Hospital, Musashino Red Cross Hospital, Osaka National Hospital in Japan and Queen Mary Hospital in Hong Kong. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the Ethics Committees of the institutions, and an informed consent was obtained from each carrier.

2.2. Serological assays for HBV markers

HBeAg and anti-HBe were detected by Chemiluminescent enzyme immunoassay (CLEIA) (Lumipulse f, FUJIREBIO INC., Tokyo, Japan). HBV Genotypes were determined by enzyme-linked immunosorbent assay with monoclonal antibodies directed to distinct epitopes on the preS2-region [20,21], with use of commercial kits (HBV GENOTYPE EIA; Institute of Immunology Co., Ltd., Tokyo, Japan).

2.3. PCR-RFLP for distinguishing between subgenotypes C1/Cs and C2/Ce of HBV genotype C

Nucleic acids were extracted from 100 μL of serum using QIAamp DNA Blood Mini Kit (Qiagen Inc., Hilden, Germany). A novel method for specific determination of HBV/C consisted of two PCR cycles with hemi-nested primers followed by RFLP with the restriction site specific for HBV/C1/Cs or C2/Ce [10]. The first-round PCR was performed with a sense primer (HBV964F) and an antisense primer (HBV1272R) within non-overlapping polymerase region. The second-round PCR was performed with a sense primer (HBV970F2) and an antisense primer (HBV1272R). To determine HBV/C1/Cs, a portion (5 μL) of the amplification product of 309 base pairs (bp) in size was digested with 5 U of *Ase*I at 37 °C and *Bst*EII at 60 °C for 1 h each. For HBV/C2/Ce digestion, *Nci*I was used at 37 °C for 2 h. Digests with these enzymes were run on electrophoresis in 3.0% (wt/vol) agarose gel, stained with ethidium bromide and examined for their sizes under the ultraviolet light.

2.4. Detection and quantification of serum HBV DNA

HBV DNA sequences spanning the S gene were amplified with real-time detection polymerase chain reaction (RTD-PCR) according to the method of Abe et al. [22] with a forward primer (HBSF2), a reverse primer (HBSR2), and Taq Man probe (HBSP2′) with an additional G at the 3′-end of the original HBSP2 [23]. The detection limit of this method was 100 copies/mL.

2.5. Amplification and sequencing of the core promoter as well as the precore region plus core gene

To confirm the results by PCR-RFLP, HBV DNA sequences bearing the core promoter and precore/core regions were amplified by PCR with hemi-nested primers by the method described previously [24], with slight modifications [23]. PCR products were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, CA) in the ABI 3100 DNA automated sequencer. The sequences covered enhancer II/core promoter (Fig. 1A) and precore genes (Fig. 1B), which could be associated with HBeAg production, viral replication and disease progression.

2.6. A cross-sectional control study for clinical and virological differences between HBV/C1/Cs and C2/Ce

The clinical diagnosis was established after serum biochemistry tests ultasonography, computerized tomography (CT), the magnetic resonance imaging (MRI), and the liver biopsy. To compare the clinical differences between HBV/C1/Cs ($n = 118$) and C2/Ce ($n = 210$), age-, sex-, HBeAg status-matched HBV carriers were enrolled (Table 1). The carriers were also matched according to the severity of liver disease in each group. The HBsAg-positive individuals with normal alanine aminotransferase (ALT) levels over 2 years (examined at least four times at 3-month intervals), and without the presence of portal hypertension were defined as IC. Individuals with persistent elevation of ALT levels (>1.5× upper limit of normal) [35 U/L] over

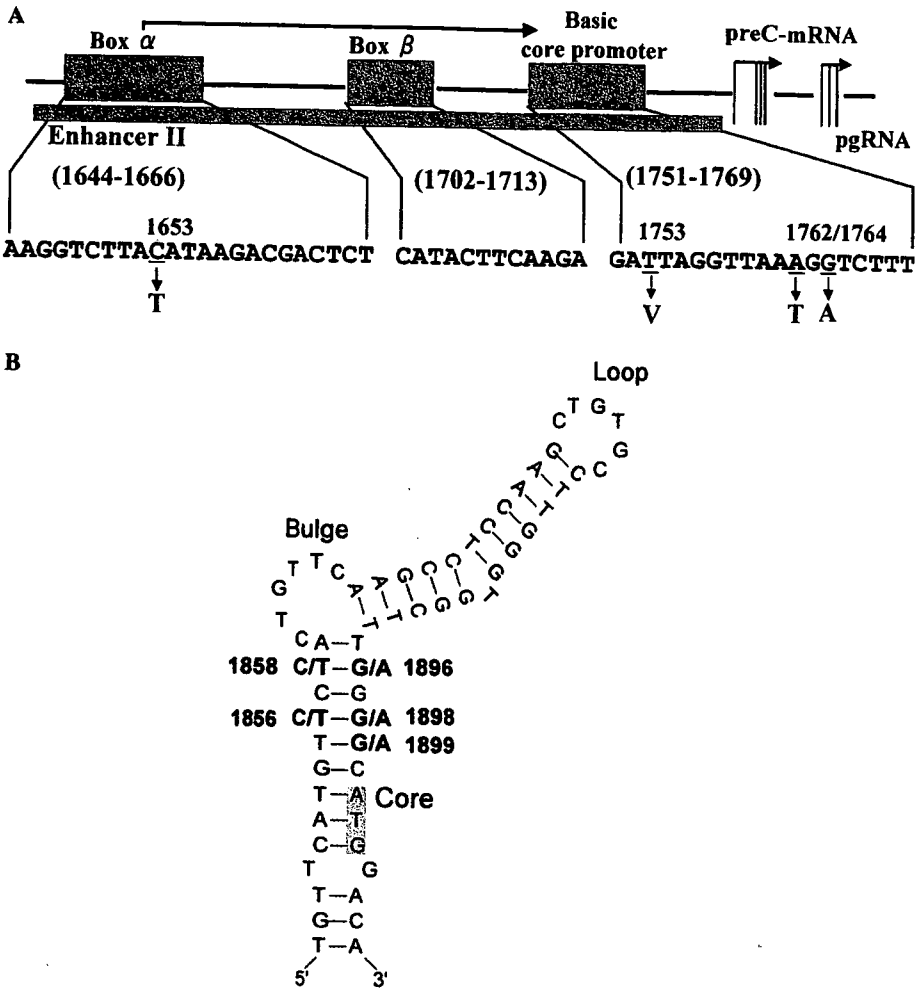

Fig. 1. (A) C1653T, T1753V (not T), A1762T/G1764A mutations in the enhancer II/core promoter region, and (B) ε loop structure of the encapsidation signal in precore genome. pgRNA, pregenomic RNA.

a 6-month period (at least three readings at 2-month intervals) without decrease of platelet count, albumin and hypersplenism (splenomegaly on ultrasonography) were defined as CH. Patients with established hepatocellular carcinoma according to the clinical biochemical investigation [Alpha-fetoprotein (AFP) and/or serum protein induced by vitamin K absence named antagonist II (PIVKA-II)], CT and/or MRI, and liver biopsy were included in HCC group. Patients with hepatitis C virus or human immunodeficiency virus co-infection were excluded, and none had received antiviral treatment during the follow-up period.

2.7. Statistical evaluation

Statistical analyses were performed using chi squared test and Fisher's exact test for categorical variables. Mann–Whitney *U* test was used for continuous variables where appropriate. The univariate general linear modeling (GLM) was used to test the effects of specific HBV mutations on the HCC and non-HCC groups of the HBV-carriers in the context of their HBeAg status. Multivariate analyses with logistic regression were used to determine the independent factors associated with

Table 1
Demographic and clinical characteristics of patients with HBV subgenotype C1/Cs and C2/Ce who were matched for age, sex, HBeAg status and clinical states

Characteristics	C1/Cs ($n = 118$)	C2/Ce ($n = 210$)	*P* value
Country (Japan/Hong Kong)	0/118	172/38	<0.0001
Age, mean ± SD	48.0 ± 13.7	50.2 ± 10.7	Matched
Sex, male (%)	96 (81%)	163 (78%)	Matched
HBeAg positive (%)	47 (40%)	62 (30%)	Matched
HBV DNA (log copies/mL), mean ± SD	5.7 ± 1.7	5.4 ± 1.4	NS
ALT (U/L), median (range)	51 (4–1154)	46 (8–773)	NS
IC/CH/HCC*	19/47/52	29/85/96	Matched
HCC (%)	52 (44%)	96 (46%)	Matched

* IC, inactive carriers; CH, chronic hepatitis; HCC, hepatocellular carcinoma; NS, not significant.

HCC. Differences were considered significant for P values less than .05. All statistical analyses were performed using the Statistical Program for Social Sciences (SPSS 12.0 for windows, SPSS Inc., Chicago, IL).

3. Results

For comparative analyses, HBV/C1/Cs ($n = 118$) and C2/Ce ($n = 211$) carriers' groups were matched in respect to age, sex, HBeAg status, and clinical states. Table 1 shows comparative summary for HBV/C1/Cs and C2/Ce groups according to the origin country of the enrolled carriers, age, sex, HBe Ag status, HBV DNA levels, ALT levels and clinical states (IC/CH/HCC); no significant differences demonstrated with exception for the original country. HBV/C1/Cs is found in Hong Kong, whereas HBV/C2/Ce is predominant in Japan.

When HCC patients in HBV/C1/Cs ($n = 52$) and C2/Ce ($n = 96$) groups were compared, no significant difference was observed in mean age, sex, HBeAg positivity, HBV DNA, and ALT levels (Table 2). However, the frequency of C-to-T1653 mutation in the box alpha (Fig. 1A) was significantly higher in HBV/C2 (C1/Cs = 23%, C2/Ce = 48%, $P = 0.0055$), whereas T-to-V1753 mutation was significantly prevalent in HBV/C1/Cs (C1/Cs = 75%, C2/Ce=39%, $P < 0.0001$). The prevalence of T1762/A1764 was high in both of these groups with no significant difference (Table 2). In the precore region including encapsidation signal (ε) (Fig. 1B), the precore stop mutation (A1896), accompanied by a C-to-T substitution at nt 1858 forming a base pairing within ε structure, was frequently found in HBV/C2/Ce strains (40/96, 42%), whereas another precore mutation (A1898), accompanied by a C-to-T substitution at nt 1856, was found only in HBV/C1/Cs strains (18/52, 35%) (Table 2). A1899 mutation was prevalent in HBV/C2/Ce (C1/Cs, 12% vs. C2/Ce, 27%) as well as A1896 mutation.

As the above mutations in the enhancer II/core promoter and precore regions could be associated with subgenotypes as well as HBeAg status, we examined the mutations predictive for HCC among all HBV/C1/Cs and C2/Ce patients in the context of HBeAg status. The prevalence of HBV mutations such as C1653T, T1753V, A1762T/G1764A and G1896A was compared among HBeAg-positive, and -negative patients with and without HCC within the C1/Cs and C2/Ce groups (Table 3). As summarized in Table 3, V1753 was frequently found among HCC patients infected with C1/Cs, when compared to those without HCC. Interestingly, the difference was greater in HBeAg-positive group ($P = 0.0055$), whereas in HBeAg-negative group the trend was only remaining ($P = 0.051$). When C2/Ce infected patients with and without HCC were compared, T1653 was frequently found among HCC patients in both HBeAg-positive and -negative groups ($P = 0.018$, 0.012, respectively), and V1753 or T1762/A1764 was also frequent in HBeAg-negative group ($P = 0.046$, 0.024, respectively). The univariate GLM confirmed the above results; V1753 mutation was predictive for HCC in the HBeAg-positive-C1/Cs ($P = 0.0092$), T1653 mutation in the HBeAg-positive-C2/Ce ($P = 0.0056$), both T1653 ($P = 0.0046$) and V1753 mutations ($P = 0.016$) in the HBeAg-negative-C2/Ce group. On the other hand, A1896 mutation was negatively correlated with HCC ($P = 0.0015$).

The factors possible attributable for HCC; age, sex, HBeAg positivity, HBV DNA level, ALT (two groups divided by median values), subgenotypes and mutations; T1653, V1753, T1762/A1764, T1856, T1858, A1896, A1898 and A1899 were tested in multiple logistic regression analysis for all 328 HBV-carriers (Table 4). Age

Table 2
Clinical and virologic characteristics of HCC patients infected with HBV subgenotype C1/Cs and C2/Ce

Characteristics	C1/Cs ($n = 52$)	C2/Ce ($n = 96$)	P value
Age, mean ± SD	54.2 ± 11.9	53.7 ± 9.0	NS
Sex, male (%)	43 (83%)	81 (84%)	NS
HBeAg-positive (%)	24 (46%)	36 (38%)	NS
HBV DNA (log copies/mL), mean ± SD	5.5 ± 1.6	5.2 ± 1.4	NS
ALT (U/L), median (range)	50 (4–1154)	53 (16–473)	NS
Mutation in the box alpha			
T1653	12 (23%)	46 (48%)	0.0055
Mutations in the core promoter			
V(not T)1753	39 (75%)	37 (39%)	<0.0001
T1762/A1764	47 (90%)	88 (92%)	NS
Mutation in the precore region			
T1856	21 (40%)	0	<0.0001
T1858	2 (4%)	96 (100%)	<0.0001
A1896	2 (4%)	40 (42%)	<0.0001
A1898	18 (35%)	0	<0.0001
A1899	6 (12%)	26 (27%)	0.05

NS, not significant.

Table 3
HBV/C1 and C2 mutations among patients with and without HCC in context of HBeAg

No.	T1653	V1753	1762/A1764	A1896
C1/CS				
HBeAg (+)				
Non-HCC ($n = 23$)	2 (8.7%)	7 (30.4%)	16 (69.6%)	4 (17.4%)
HCC ($n = 24$)	6 (25.0%)	**18 (75.0%)***	22 (91.7%)	1 (4.2%)
HBeAg (−)				
Non-HCC ($n = 43$)	8 (18.6%)	21 (48.8%)	33 (76.7%)	10 (23.2%)
HCC ($n = 28$)	6 (21%)	21 (75%)	25 (89%)	1 (4%)
C2/Ce				
HBeAg (+)				
Non-HCC ($n = 26$)	1 (3.8%)	7 (26.9%)	17 (65.4%)	6 (23.1%)
HCC ($n = 36$)	**10 (27.8%)**	15 (41.7%)	31 (86.1%)	9 (25.0%)
HBeAg (−)				
Non-HCC ($n = 88$)	33 (37.5%)	18 (20.5%)	71 (80.7%)	57 (64.8%)
HCC ($n = 60$)	**36 (60.0%)***	**22 (36.7%)***	**57 (95.0%)***	31 (51.7%)

* Non-HCC vs. HCC, $P < 0.05$ (Yates corrected chi-square). Significant data are shown in bold.

($\geqslant 50$) [odds ratio (95% CI): 2.90 (1.72–4.89), $P < 0.0001$], sex (male) [2.29 (1.18–4.43), $P = 0.014$], HBeAg (positive) [2.39 (1.34–4.28), $P = 0.003$] and subgenotype (C2/Ce) [4.21 (1.07–16.23), $P = 0.039$] were significantly associated with the development of HCC. HBV mutations found in strong association with HCC were T1653 [3.64 (1.93–6.86)], V1753 [3.07 (1.66–5.65)], and T1762/A1764 [2.58 (1.21–5.49)]. A1896 stop-codon mutation was negatively correlated with HCC [0.31 (0.16–0.62), $P = .001$] in this population (Table 4).

4. Discussion

In this study, we focused on HBV/C, which is prevalent in Asia and possibly contribute to progressive liver disease and poor clinical outcomes in HBV-carriers [3,25]. Previous reports containing epidemiological and phylogenetic analyses of the HBV/C complete genome determined at least 4 subgenotypes (C1–4) with different geographic distribution [10,26]. HBV/C1 was found only in Southeast Asia, and HBV/C2 was found in Far East Asia; while remaining two were rarely found in most Asian countries, and probably represent isolated local epidemics; HBV/C3 found in Pacific countries and HBV/C4 strains were isolated from Aborigines in Northeast Australia [24]. In the present study, we examined the clinical and virological differences between C1/Cs and C2/Ce.

The multivariate analysis in this study showed that the following factors were predictive for HCC; subgenotype C2/Ce, and mutations in the enhancer II/core promoter; T1653, V1753 and T1762/A1764. In agreement with the previous reports [27–29], the elder age ($\geqslant 50$), male sex and HBeAg positive were also independent risk factors for HCC. The T1653 and V1753 mutations had been first reported by Takahashi et al. [17]; these specific mutations were prevalent among Japanese HCC patients. Our recent age-, sex-matched case-control study also confirmed that the T1653 mutation in the box alpha in addition to the T1762/A1764 double mutation increases the risk of HCC in anti-HBe-positive patients with HBV/C [19]. The T1762/A1764 mutation had been highly frequent in the elder HBV/C carriers ($\geqslant 50$) regardless of the clinical states [19]; however, these results do not contradict that T1762/A1764 is associated with hepatocarcinogenesis, because poor prognosis of HBV/C compared to HBV/B (Ba and Bj) correlated with high prevalence of T1762/A1764 [3,7,11]. A prospective cohort of 1638 high-risk individuals in Qidong (China) showed that the T1762/A1764 mutation was detected in 8 of the 15 HCC cases (53.3%) before cancer [30], suggesting that the T1762/A1764 double mutation would indicate a high potential risk for hepatocarcinogenesis. Hence, T1653 and/or V1753 mutations in addition to T1762/A1764 are strongly associated with HCC development.

Buckwold et al. reported that the emerging T1762/A1764 dramatically decreases the affinity with the liver-enriched transcription factors resulting in the reduction of the HBeAg expression [31]. Thereafter, Li et al. reported that this mutation not only affects the nuclear receptor binding site but also creates a new HNF1 transcription factor binding site [32]. In the previous study, it was demonstrated that the box alpha elements (1646–1668) individually stimulate the promoter activity for more than 100-fold [16]. The T1653 mutation converts the alpha box binding site for C/EBP and related factors into the perfect palindromic sequence 1648-TCTTATATAAGA, which might enhance binding affinity and enhancer II/core promoter activity. Hence, the T1653 mutation could influence the HBeAg production and viral replication through the BCP activity. Although a number of studies have reported the role of the BCP mutations in the viral features, the exact mechanisms of HCC development still remains unclear,

Table 4
Variables with independent predictive value for HCC in the multivariate analysis

Factor	Total ($n = 328$)	
	Odds ratio (95% CI)	P value
Age*		
<50	1	
⩾50	2.90 (1.72–4.89)	$P < 0.0001$
Sex		
Female	1	
Male	2.29 (1.18–4.43)	$P = 0.014$
HBeAg		
Negative	1	
Positive	2.39 (1.34–4.28)	$P = 0.003$
HBV DNA (log copies/ml)*		
<5.5	1	
⩾5.5	0.74 (0.25–2.86)	NS
ALT (U/L)*		
<50	1	
⩾50	1.75 (0.56–6.63)	NS
HBV subgenotypes		
C1/Cs	1	
C2/Ce	4.21 (1.07–16.23)	$P = 0.039$
T1653 mutation		
Absence	1	
Presence	3.64 (1.93–6.86)	$P < 0.0001$
V(not T)1753 mutation		
Absence	1	
Presence	3.07 (1.66–5.65)	$P < 0.0001$
T1762/A1764 mutation		
Absence	1	
Presence	2.58 (1.21–5.49)	$P = 0.014$
T1856 mutation		
Absence	1	
Presence	0.41 (0.10–1.69)	NS
T1858 mutation		
Absence	1	
Presence	0.32 (0.07–1.43)	NS
A1896 mutation		
Absence	1	
Presence	0.31 (0.16–0.62)	$P = 0.001$
A1898 mutation		
Absence	1	
Presence	3.31 (0.72–15.35)	NS
A1899 mutation		
Absence	1	
Presence	1.14 (0.57–2.29)	NS

* Two groups were divided by each median value. NS, not significant.

particularly in respect to reflection of the mutations on the X protein. The T1653 mutation responsible for an amino acid change from histidine to tyrosine at aa 94 of the X protein, so this alteration of X protein might be somehow associated with hepatocarcionogenesis. Similarly, the changes of amino acids from isoleucine to asparagine/serine/threonine by V1753 mutation may also affect the function of X protein.

Many previous studies on the HBV encapsidation sequence focused on the configuration of nucleotide 1858 [33,34]. Of note, all HBV/C2/Ce strains possessed T1858 and most HBV/C1/Cs had C1858. A previous study carried among multi-ethnic carriers in Hawaii indicated no significant difference in clinical characteristics between C1858 and T1858 variants [35]. Although the polymorphism of C or T at nucleotide 1858 affects the development of the precore stop-codon mutation, it does not seem to influence disease activity [4,11,36,37]. A recent report showed that HBV carriers bearing TCC at nucleotides 1856–1858 had higher HBeAg positivity and ALT levels than those with CCT; but similar prevalence of liver cirrhosis was observed between them [37]. The precore stop-codon mutation (A1896), accompanied by a C-to-T substitution at nt 1858 forming a base pair with it, was found mainly in HBV/C2/Ce, and another precore mutation (A1898), accompanied by a C-to-T mutation at nt 1856, was found only in HBV/C1/Cs strains. These mutations could stabilize the ε loop structure and the former HBeAg-negative mutants bearing a TAG stop-codon mutation at codon 28 (A1896) uniformly replicate at least 20-fold better than mutants bearing a TGA stop-codon at the same amino acid position enhance viral replication [38]. Although the stringent selection for a highly efficient RNA encapsidation element may play a crucial role in the natural occurrence of these two closely linked precore mutations, the multivariable analysis in this study showed that A1896 stop-codon mutation was negatively correlated with HCC development. The result remains controversial [36,39,40] and the mechanism of HCC development in association with A1896 mutation remains unclear.

HBeAg positivity was one of independent predictive factor for HCC in this study, which was consistent with a previous prospective study by Yang et al. [41]. The biologic function of HBeAg remains controversial. HBeAg is not required for viral replication; but it appears to be necessary for the establishment of chronic infection in animal models [42]. The most common mutation in the precore sequence that abrogates the synthesis of HBeAg is a stop-codon mutation (G1896A). As all HBV/C2/Ce strains possessed T1858 and most C1/Cs had C1858, the C1/Cs with C1858 might be responsible for a delayed seroconversion for the loss of HBeAg in the carriers of C1/Cs [37]. The mechanisms of HBeAg seroconversion and its association with HCC development remain unclear. Assuming that different mechanism may exist leading to carcinogenesis in context of HBeAg status of patients, in the present cross-sectional control study, we examined patients divided into four subgroups in respect to subgenotypes/HBeAg status. In univariate analysis the V1753 mutation was confirmed as predictor for HCC in the HBeAg-positive-C1/Cs, and T1653 mutation in the HBeAg-positive-C2/Ce. Interestingly, in the HBeAg-negative-C2/Ce group, T1653 or V1753 or T1762/A1764 mutations