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＜速　報＞

核酸アナログ療法中のB型関連肝癌に対する肝癌再発予測マーカーとしてのHBコア関連抗原の有用性

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緒言：B型肝疾患に対する核酸アナログ療法の有効性は広く知られており，ラミブジンにおいては投与により発癌率を抑制することが既に報告されている[1)2)]．しかしながら経過観察期間が長くなるにつれ肝発癌例も増加しつつある．また血中HBV-DNA量が抑制されているにもかかわらず，肝癌根治後の再発例も散見される．そこで今回我々は核酸アナログ投与中の肝癌について，肝癌根治療法後の再発予測マーカーとしてのHBコア関連抗原（HBcrAg）の有用性を検討した．

対象と方法：2001年～2008年までに当院で初発の肝細胞癌と診断されたB型肝癌症例で核酸アナログ投与中に肝発癌した54例を対象とした．肝癌発症時の核酸アナログ投与内容の内訳はラミブジン29例，ラミブジン＋アデフォビル併用17例，エンテカビル8例であった．肝癌治療法の内訳は外科切除36例，経皮的局所治療18例であった．HBcrAg測定は既報のごとくCLEIA法を[3)]，HBV-DNA量はアンプリコア法を用いた．肝癌根治後の再発に寄与する因子についてCox比例ハザードモデルを用いて，単変量及び多変量解析を行い検討した．

結果：発癌時のAST/ALT値は31/29 IU/l(中央値)，genotype Cが92.6%（50/54）で，HBe抗原陽性例は42.6%（23/54），血清HBV-DNA量は＜2.6 log copies/ml（中央値）であった．血清HBcrAg量は5.0 logU/ml（中央値）であった．血清HBV-DNA量＜2.6 log copies/mlであった症例35例中，HBcrAg量≧3.0 logU/mlであった症例が29例（82.9%），≧4.8 logU/mlであった症例は13例（37.1%）であった．核酸アナログ投与開始から発癌までの投与期間は2.2年（中央値）であった．

肝癌再発は38.9%（21/54）で認め，根治後から再発までの期間は14カ月（中央値）であった．再発に寄与する因子について単変量解析を行ったところ，HBV-DNA量≧3.0 log copies/ml，HBcrAg≧4.8 logU/ml，腫瘍数多発，門脈浸潤ありの4因子が抽出され，さらに多変量解析を行ったところ，独立因子としてHBcrAg≧4.8 logU/ml，門脈浸潤の2因子が抽出された(Table)．

考察：今回の検討では核酸アナログ投与中の発癌例は血清HBV-DNA量が低値に抑制されているにもかかわらず，HBcrAg量は十分抑制されていない例が認められた[4)]．核酸アナログが投与されていないB型肝癌において，血清HBV-DNA量が肝癌再発に関係するという報告はされている[5)]．しかしながら今回の対象症例のように核酸アナログ投与中の場合はHBV-DNA量よりHBcrAg量の方が肝癌根治後の再発予測マーカーとして有用であると考えられる．

索引用語：HBコア関連抗原，肝癌再発予測，核酸アナログ

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Table Factors associated with recurrence of HCC by univariate and multivariate analysis.

	Univariate		Multivariate	
factors	Hazard Ratio (95%CI)	P	Hazard Ratio (95%CI)	P
HBeAg (Positive)	1.53 (0.63-3.70)	0.343		
HBV DNA ($\geq$ 3.0 logcopies/mL)	2.49 (1.03-6.00)	0.042		
HBcrAg ($\geq$ 4.8 logU/mL)	10.4 (2.39-45.0)	0.002	8.50 (1.95-37.1)	0.004
AST ($\geq$ 50 IU/L)	2.47 (0.98-6.20)	0.055		
ALT ($\geq$ 40 IU/L)	2.37 (0.99-5.71)	0.054		
Platelets count ($< 10^5$ /mm^3)	2.20 (0.81-6.02)	0.123		
Serum Albumin ($<$ 3.5 g/dl)	1.39 (0.53-3.63)	0.505		
Serum bilirubin ($\geq$ 1.5 mg/dl)	1.11 (0.62-2.00)	0.713		
Prothorombin time ($<$ 80%)	2.23 (0.51-9.82)	0.286		
ICG-R 15 ($\geq$ 30%)	0.54 (0.16-1.87)	0.332		
AFP levels ($\geq$ 100 ng/mL)	1.81 (0.74-4.44)	0.194		
DCP levels ($\geq$ 100 mAU/mL)	2.09 (0.81-5.39)	0.129		
Tumor size ($\geq$ 21 mm)	2.02 (0.81-5.07)	0.133		
Tumor number (multiple)	4.03 (1.31-12.4)	0.015		
Presence of portal vein invasion	5.39 (1.69-17.2)	0.004	3.63 (1.15-11.5)	0.028

Abbreviation: AST, aspartate aminotransferase; ALT, alaine aminotransferase; ICG-R15: indocyanine green retention test at 15 min; AFP, alpha-fetoprotein; DCP, des-γ-carboxylprothorombin,

英文要旨

Low hepatitis B virus core-related antigen is a predictor of absence in post-treatment recurrence of hepatocellular carcinoma during antiviral therapy

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The tumor recurrence rate of hepatocellular carcinoma (HCC) is still high even in patients who receive a curative therapy. We analyzed predictive value of HBV-related viral markers, including HBcrAg, HBV DNA, and HBeAg, for HCC recurrence in the patients who developed HCC during antiviral nucleot(s)ide analogues therapy. By univariate analysis, HBV DNA, HBcrAg, tumor number and presence of portal vein invasion were significant predictive factors. By multivariate analysis, HBcrAg and presence of portal vein invasion were independent and significant predictive factors of recurrence after curative therapy for HCC. We conclude that HBcrAg is useful as a predictor of post-treatment recurrence of HCC after curative therapy in patients who received antiviral therapy.

Key words: HB core-related antigen, prediction of recurrence of HCC, nucleot(s)ide analogues

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INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

The Efficacy of Interferon-beta Monotherapy for Elderly Patients with Type C Hepatitis of Genotype 2

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Abstract

Objective The aim of this study was to elucidate the efficacy of interferon (IFN)-beta monotherapy for elderly patients of ≥70 years with type C hepatitis (HCV) of genotype 2.

Methods The present study was a retrospective cohort study. Inclusion criteria were type C hepatitis patients with HCV genotype 2a or 2b, ≥70 years, and IFN-beta monotherapy of within 24weeks. Thirty-one consecutive patients who satisfied the above criteria were enrolled in the present study. Independent factors that might have influenced the sustained virological response (SVR) were studied using logistic regression analysis.

Results Background of clinical profiles was as follows: median (range) age =71 (70-76) years, male/female =13/18, and median (range) HCV-RNA=260 (<5-3,800) KIU/mL. Out of 31, 16 patients (51.6%) had SVR by the intention-to-treat analysis. The SVR was significantly associated with the serum HCV RNA level. Logistic analysis showed that SVR occurred when HCV RNA level was <100 KIU/mL (p=0.020). Based on the difference of the serum HCV RNA level, the SVR rate was 81.8% (9/11) in patients with a serum HCV RNA level of <100 KIU/mL and 35.0% (7/20) in patients with a serum HCV RNA level of ≥100 KIU/mL.

Conclusion IFN-beta monotherapy of ≤24 week is a possible therapy selection for elderly patients of ≥70 years with type C hepatitis of genotype 2.

Key words: elderly patients, hepatitis C virus, genotype 2a or 2b, interferon monotherapy, sustained virological response

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Introduction

Current interferon (IFN) therapy for patients with chronic hepatitis C viral (HCV) infection has been directed at viral clearance. Recent studies have reported improvement of therapeutic efficacy when IFN is combined with ribavirin (1-6). However, IFN is expensive and has a number of serious side effects. The adverse events have a tendency to occur in elderly patients (7, 8). Therefore, in the case of elderly patients, the physician in charge often avoids IFN therapy because of IFN side effects. However, recently, the lifespan has been long in Japan. Thus, in the near future, a large number of patients with HCV will be >60 years of age. Also, HCV-related hepatocellular carcinoma (HCC) patients have been shown to become old with a peak around age 70 (9-11). When such aged patients with chronic abnormal ALT levels consult a doctor, the decision of whether or not to use therapy for chronic hepatitis is problematic. Moreover, when the use of treatment for chronic hepatitis C is decided for such aged patients, whether or not IFN therapy should be second problem.

A few studies have targeted IFN therapy and prolonged prognosis in elderly patients with chronic hepatitis C. Our

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investigation showed that the clearance of hepatitis C virus reduces the onset of HCC in elderly patients with HCV (12). Imai et al reported that IFN therapy reduces liver-related mortality in aged patients with chronic hepatitis C, especially in those exhibiting a biochemical response as well as a sustained virological response (13). Thus, in Japan, elderly patients with HCV are often treated with IFN.

In IFN therapy for chronic hepatitis C, several predictive factors of sustained virological response (SVR) to IFN have been identified, and these include short duration of disease, young age, absence of liver cirrhosis, genotype 2, low HCV-RNA levels, HCV and mutant type of nonstructual5A region (14-18). Thus, HCV patients with genotype 2 or low HCV-RNA levels might have the possibility of eradication of HCV RNA with a small dose or a short period of IFN. Now, there is also controversy about the indication and administration method of the IFN therapy in elderly patients with HCV.

Thus, in this study, we evaluated the efficacy of interferon (IFN)-beta monotherapy for type C patients of ≥70 years with genotype 2.

Abbreviation: ALT: alanine aminotransferase, AST: aspartate aminotransferase, CH: chronic hepatitis, HCV: hepatitis C virus, IFN: interferon, LC: liver cirrhosis, MU: million unit, SVR: sustained virological response

Materials and Methods

Patients

A total of 31 consecutive cirrhotic type C patients treated with IFN-beta for HCV RNA clearance at Toranomon Hospital in Tokyo, Japan between 2000 and 2007 were enrolled in this study. This study was a retrospective cohort study. Enrollment criteria were: ≥70 years; positive serum HCV RNA; genotype 2a or 2b; IFN-beta monotherapy; treatment period of ≤24 weeks. We excluded from the study all of the following patients: 1) those with concurrent hepatitis B virus (HBV); 2) with a history of IFN therapy; 3) leukocytes <3,000/mm^3, platelets <70,000/mm^3 and bilirubin >1.5 mg/mL before IFN therapy; 4) decompensated liver cirrhosis with ascites or encephalopathy.

IFN therapy

For the first IFN treatment regimen, the IFN treatment consisted of 3 to 6 million units (MU) of IFN-beta (Toray Industries or Daiichi Pharmaceutical Co., Tokyo, Japan). For the IFN treatment regimen, one group of 20 patients was given to receive IFN-beta intravenously every day for the first 2-8 weeks and then two or three times a week for the following 16-22 weeks (long-term group). Another group of 11 patients were treated with IFN by intravenous injection daily for 6-8 weeks (short-term group). The physician in charge primarily determined the method of IFN treatment and dose of IFN. We regarded sustained virological response (SVR) to therapy as clearance of HCV RNA by amplicor method (19) for more than 6 months after cessation of therapy. Our study was approved by the institutional ethics review board of our hospital. The physician in charge explained to each patient the purpose and method of the treatment as well as the potential adverse reactions, and informed consent for treatment was then obtained.

Blood testing

Blood samples were obtained just before IFN therapy and stored at -80℃. Using these blood samples, HCV-RNA levels before IFN therapy were analyzed by quantitative PCR assay (Amplicor GT-HCV Monitor Version 2.0, Roche Molecular Systems, Branchburg, NJ, USA) (20).

On the other hand, serum HCV-RNA at 6 months after the termination of IFN therapy was analyzed by the qualitative PCR assay (19). The lower detection limit of the qualitative assay is 100 copies/mL. HCV genotype was examined by the PCR assay, using a mixture of primers for the six subtypes known to exist in Japan, as reported previously (21).

Liver staging

Ideally, the severity of chronic liver disease should be determined histologically from the results of liver biopsy. Only 12 (38.7%) of 31 patients underwent peritoneoscopy or liver biopsy before the age of 70; the remaining 19 patients did not undergo histological assessment on the first visit owing to their advanced age. In these patients, liver staging was determined by calculation using the equation to discriminate chronic hepatitis (CH) and liver cirrhosis (LC) as described by Ikeda et al (22).

Statistical analysis

Nonparametric procedures were employed for the analysis of background features of the patients with SVR and without SVR, including the Mann-Whitney U test. Independent factors that might have influenced SVR were studied using multiple logistic regression analysis, and the following variables were evaluated as prognostic factors: sex, age, body mass index, HCV RNA level, HCV genotype 2a or 2b, liver staging, biochemical factors (AST, ALT), platelet count, total IFN dose, and IFN regimen. The SPSS software package (SPSS Inc., Chicago, IL) was used to perform statistical analysis. A p value of <0.05 was considered to indicate a significant difference.

Abbreviation: AST: aspartate aminotransferase

Results

Patients' characteristics

Table 1 shows the characteristics of the 31 patients who had received IFN-beta monotherapy. Clinical profiles were as follows: median (range) age =71 (70-76) years, male/female =13/18, median (range) HCV-RNA=260 (<5-3,800) KIU/mL, and CH/LC =19/12. All LC patients were catego-

Table 1. Clinical Characteristics before Interferon Therapy for Elderly Patients with Hepatitis C Virus of Genotype 2

Characteristics	(n=31)
Age (years old)	71(70-76)
Male/female	13/18
Body mass index	21.6 (17.3-25.4)
Complication of diabetes mellitus	0/31 (0%)
Complication of hypertension	4/31 (12.9%)
IFN therapy (short-regimen/long-regimen) *	11/20
Total dose of IFN (MU)	336 (12-624)
Liver Staging (CH/LC)	19/12
HCV load (KIU/mL)	260 (<5-3.800)
HCV genotype (2a/2b)	20/11
AST (IU/L)	55 (18-141)
ALT (IU/L)	65 (14-255)
Platelet (10^4/mm^3)	13.4 (7.3-21.6)
SVR	16/31 (51.6%)

ALT, alanine aminotransferase;
Data are expressed as number of patients or median (range)
* One group of 20 patients was given to receive IFN-beta intravenously every day for the first 2-8 weeks and then two or three times a week for the following 16-22 weeks (long-term group). Another group of 11 patients were treated with IFN by intravenous injection daily for 6-8 weeks (short-term group).

rized as Child-Pugh-Turcotte score class A.

Safety and tolerance in IFN group

Of the 31 patients originally included in this study, three discontinued IFN therapy due to owing to adverse events: that is, one patient each of nausea on the 3rd day after the initiation of IFN, general fatigue on the 7th day, and poor appetite at the 22nd week. On the other hand, for four patients the dose of the IFN therapy was reduced from 6 MU to 3 MU because of general fatigue and thrombocytopenia at 3-8 weeks after the initiation of IFN. Of these four patients one was in the short-term regimen and three in the long-term regimen. Thus, the median total dose was 336 MU (range, 12-624MU).

Efficacy of treatment

Out of 31 patients enrolled on the present study, 16 patients (51.6%) had SVR by the intention-to-treat analysis. The SVR was significantly associated with serum HCV RNA level. The patients with a HCV RNA level of <100 KIU/mL tended to have a high SVR compared to those with a HCV RNA level of ≥100 KIU/mL (p=0.020) (Table 2). Based on the difference of the serum HCV RNA level, the SVR rate was 81.8% (9/11) in patients with a serum HCV RNA level of <100 KIU/mL and 35.0% (7/20) in patients with a serum HCV RNA level of ≥100 KIU/mL. Serum HCV RNA at 4 week after the initiation of IFN could be determined in twenty-nine patients. The negativity rate of serum HCV RNA at 4 week after the initiation of IFN was 76.2% (16/21) in the SVR group and 0% (0/8) in the non-SVR group. Table 3 shows the differences in the clinical background between patients with SVR and those without SVR. The serum level of HCV RNA in patients with SVR was lower than that in patients without SVR. Table 4 shows the SVR rate based on the HCV load and IFN regimen. Table 5 shows the SVR rate based on the HCV load and the total dose of IFN. In patients with low virus load, the SVR rate in patients treated by the short-term regimen or a total dose of IFN of <350 MU was almost the same as that in patients treated by the long-term regimen or a total dose of ≥ 350 MU. On the other hand, in patients with high virus load, a high total dose has a tendency to enhance the SVR.

Discussion

The present study was limited by the fact that it was a non-randomized controlled trial. Another limitation of the study was that the number of patients was small. However, several findings from the present study have direct implications for the IFN treatment of elderly patients with genotype 2a or 2b.

First, about half of the patients of genotype 2 treated with IFN-beta monotherapy cleared HCV RNA. This result indicates that the IFN monotherapy is a possible selection of therapy for elderly patients with genotype 2. Second, the patients with HCV RNA level of <100 KIU/mL tend to have high SVR compared to those with a HCV RNA level of ≥ 100 KIU/mL. On the treatment regimen, the efficacy in the short-term regimen of IFN therapy was almost the same as that of the long-term regimen in patients with low-virus load. Moreover, the efficacy of the total dose of IFN of < 350 MU did not differ from that of a total dose of ≥350 MU in patients with a low virus load. These results indicate that in about 80% of elderly patients with a genotype 2 and serum HCV RNA level of <100 KIU/mL, HCV was eradicated by the 6- to 8-week regimen or total dose of IFN of < 350 MU. On the other hand, in patients with a high virus load, a high total dose might have a tendency to enhance the SVR.

Table 2. Predictive Factors for SVR in Interferon Therapy for Elderly Patients with Hepatitis C Virus of Genotype 2

Factor	Category	Odds ratio	95% CI	p value*
HCV RNA (KIU/mL)	<100 /≥100	1/8.36	1.40-49.88	.020
AST (IU/L)	≥38 /<38	1/0.57	0.81-4.01	.573
Age (years)	<75/ ≥75	1/0.75	0.14-4.10	.740
Platelet (10^4/mm^3)	≥15/<15	1/0.64	0.15-2.77	.553
Liver staging	(CH /LC)	1/0.90	0.21-3.85	.886
Sex	Female / Male	1/1.17	0.28-4.87	.883
ALT (IU/L)	≥50/<50	1/0.60	0.13-2.78	.521
Total dose of IFN (IU/L)	≥400/<400	1/0.67	0.16-2.77	.577
IFN regimen †	long / short	1/1.67	0.60-4.66	.330
HCV genotype	2b/2a	1/4.95	0.99-24.88	.052
Body mass index	<25/≥25	1/1.08	0.18-6.44	.930

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; CI, confidence interval; HCV, hepatitis C virus; IFN, interferon; LC, liver cirrhosis; SVR, sustained virological response;

*p value calculated by logistic regression analysis, Negativity rate of serum HCV RNA at 4week after the initiation of IFN was 76.2%(16/21) in the SVR group and 0%(0/8) in the non-SVR group.

† One group of 20 patients was given to receive IFN-beta intravenously every day for the first 2-8 weeks and then two or three times a week for the following 16-22 weeks (long-term group). Another group of 11 patients were treated with IFN by intravenous injection daily for 6-8 weeks (short-term group).

Table 3. The Difference of Clinical Backgrounds between Patients with SVR and Those without SVR

	SVR (n=16)	Non-SVR (n=15)	p value *
Age (years)	70 (70-76)	71 (70-76)	0.379
Sex (male/female)	8/9	6/9	0.735
Liver staging (CH/LC)	10/6	9/6	1.000
Body mass index	21.7(17.3-25.7)	20.3(18.8-25.9)	0.766
Total dose of IFN (MU)	336 (90-624)	336 (12-624)	0.545
IFN method (short term/long term) †	6/10	5/10	1.000
HCV genotype (2a/2b)	13/3	7/8	0.066
HCV-load (KIU/mL)	120 (<5-2300)	580 (33-3800)	0.041
HCV RNA at 4 week (-/+)	16/0	7/8	<0.001
AST (IU/L)	59 (25-141)	54 (18-99)	0.313
ALT (IU/L)	65 (17-255)	59 (14-148)	0.667
Platelet (10^4/mm^3)	14.9 (7.3-21.6)	14.1 (10.4-22.0)	0.626

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; HCV, hepatitis C virus; IFN, interferon; LC, liver cirrhosis; MU, million unit; SVR, sustained virologic response;

Data are expressed as number of patients or median (range),

*p value calculated by the Mann-Whitney U test

† One group of 20 patients was given to receive IFN-beta intravenously every day for the first 2-8 weeks and then two or three times a week for the following 16-22 weeks (long-term group). Another group of 11 patients were treated with IFN by intravenous injection daily for 6-8 weeks (short-term group).

Table 4. The SVR Rate Based on the HCV Load and IFN Regimen

HCV load (KIU/mL)	IFN regimen*		Total
	Short-term (6-8 weeks)	Long-term (24 weeks)	
Low-virus load (<100)	83.3% (5/6)	80.0% (4/5)	81.8% (9/11)
High-virus load (≥100)	20.0% (1/5)	40.0% (6/15)	35.0% (7/20)
Total	54.5% (6/11)	50.0% (10/20)	51.6% (16/31)

HCV, hepatitis C virus; IFN, interferon.

* One group of 20 patients was given to receive IFN-beta intravenously every day for the first 2-8 weeks and then two or three times a week for the following 16-22 weeks (long-term group). Another group of 11 patients were treated with IFN by intravenous injection daily for 6-8 weeks (short-term group).

Table 5. The SVR Rate Based on the HCV Load and a Total Dose of IFN

HCV load (KIU/mL)	A total dose of IFN (million units)		Total
	<350	≥350	
Low-virus load (<100)	75% (6/8)	100% (3/3)	81.8% (9/11)
High-virus load (≥100)	20% (2/9)	45.4% (5/11)	35.0% (7/20)
Total	47.1% (8/17)	57.1% (8/14)	51.6% (16/31)

HCV, hepatitis C virus; IFN, interferon.

Regarding the side effects of IFN, three patients withdrew the treatment due to IFN-related side effect. Moreover, four patients had to reduce the IFN dose due to IFN side effects. For IFN therapy for elderly patients, the physician in charge should check the clinical findings compared to young patients.

At present, the combined IFN and ribavirin therapy is a standard therapy for chronic hepatitis C patients with a high load of HCV-RNA. However, prolonged combination therapy of IFN and ribavirin is associated with various side effects. If the total dose of IFN is decreased and the period of IFN therapy is short, it would be desirable from two points: cost and side effect.

IFN-beta should be given intravenously. The intravenous injection is not convenient for treatment compared to intramuscular or subcutaneous injection. However, IFN-beta-related side effects are mild and few compared to combination therapy of IFN-alpha. Katamura et al has reported that IFN-beta-induced mental disorders are milder than those induced by PEG-IFN (23). The present study indicates that patients ≥70 years old tolerate IFN-beta.

Fortunately, in patients with genotype 2 and low virus load, HCV RNA tends to be eradicated with a small dose of IFN (24-27). The present study indicates that in elderly patients of ≥70 years with a low HCV-RNA, HCV RNA can be eradicated with a low dose of IFN.

Conclusion

The present study indicates that IFN-beta monotherapy of ≤24 weeks is a possible selection of therapy for elderly patients of ≥70 years old with type C hepatitis of genotype 2.

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Losartan Reduces the Onset of Type 2 Diabetes in Hypertensive Japanese Patients With Chronic Hepatitis C

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The aim of this retrospective cohort study is to assess the cumulative development incidence and predictive factors for type 2 diabetes (T2DM) in HCV positive and hypertensive patients treated with losartan. Eighty Japanese patients were given 50 mg of losartan per day after diagnosis of hypertension (losartan group). Another 160 treated with spironolactone were selected as control (spironolactone group). Patients in spironolactone group were matched 1:2 with losartan group for age and sex. The mean observation period was 5.2 years in losartan group and 5.4 years in spironolactone group. An overnight (12 hr) fasting blood sample or a casual blood sample was taken for routine analyses during follow-up. The primary goal is the onset of T2DM. Evaluation was performed by using the Kaplan–Meier method and the cox proportional hazards analysis. Three patients in losartan group and 20 in spironolactone group developed T2DM. The 5th year cumulative appearance rates of T2DM were 5.4% in losartan group and 14.4% in spironolactone group. Multivariate cox proportional hazards analysis showed that T2DM development after the initiation of anti-hypertensive drugs occurred when anti-hypertensive drug was spironolactone (hazard ratio: 6.10; 95% confidence interval = 1.78–20.84; $P = 0.004$), histological staging was advanced (hazard ratio: 4.31; 95% confidence interval = 1.94–9.60; $P < 0.001$), fatty liver was present (hazard ratio: 3.28; 95% confidence interval = 1.47–7.27; $P = 0.004$), and patient had pre-diabetes (hazard ratio: 2.47; 95% confidence interval = 1.08–5.63; $P = 0.032$). Our results indicate losartan causes about 60% reduction of the onset of T2DM compared to patients treated with spironolactone. ***J. Med. Virol. 81:1584–1590, 2009.***

KEY WORDS: hepatitis C virus; hypertension; losartan; type 2 diabetes mellitus; a retrospective cohort study

INTRODUCTION

Hepatitis C virus (HCV) is one of the more common causes of chronic liver disease in world. Chronic hepatitis C is an insidiously progressive form of liver disease that relentlessly but silently progresses to cirrhosis and/or hepatocellular carcinoma (HCC) over a period of 10–30 years [Kiyosawa and Furuta, 1991; Alter et al., 1992; Ikeda et al., 1993; Tsukuma et al., 1993]. Additionally, data supporting a link between Type 2 diabetes mellitus (T2DM) and chronic hepatitis C

Abbreviations used: ALT, alanine aminotransferase; normal range = 11–36; AST, aspartate aminotransferase; normal range = 6–34; CI, confidence interval; FPG, fasting plasma glucose; HCV, hepatitis C virus; HR, hazard ratio.

Specific author contributions: Yasuji Arase: design, data collection, data analysis, manuscript development and oversight; Fumitaka Suzuki: design, data collection, data analysis, manuscript development; Yoshiyuki Suzuki: data collection; Norio Akuta: data collection; Masahiro Kobayashi: data collection; Yusuke Kawamura: data collection; Hiromi Yatsuji: data collection; Hitomi Sezaki: data collection; Tetsuya Hosaka: data collection; Miharu Hirakawa data collection: Kenji Ikeda: data collection; Hiromitsu Kumada: design, data collection, data analysis, manuscript development and oversight; Tetsuro Kobayashi: manuscript development and oversight.

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infection have been reported [Arao et al., 2003; Mehta et al., 2003; Romero-Gómez et al., 2008; Imazeki et al., 2008; Arase et al., 2009]. Recently, hypertension increased in chronic liver disease with increase of elderly patients in Japan. Administration of losartan has been proven to be useful for the treatment of hypertension [Dahlöf et al., 2002; Lindholm et al., 2002]. Some previous studies have presented conflicting results with some suggesting that angiotensin receptor antagonist improves insulin sensitivity and exert beneficial effects on glucose and lipid metabolism [Iimura et al., 1995; Yusuf et al., 2000; Ando and Fujita, 2006]. Whereas others found that losartan did not influence insulin sensitivity [Fogari et al., 1998]. These discrepancies might depend on factors such as race, age, stage of hypertension, structural vascular changes in precapillary arteries. However, in any case, there is little information on the yearly cumulative incidence and risk factors on the development rate of T2DM in hypertensive patients with type C chronic liver disease during the prolonged follow-up.

In Toranomon Hospital (Tokyo, Japan), the authors evaluate a large number of patients with HCV-related hepatitis, and often find hypertension and T2DM. With this background in mind, the cohort study was initiated to investigate the cumulative incidence and risk factors of T2DM after prolonged follow up in HCV-infected and hypertensive patients treated with antihypertensive drugs. The strength of the current study is the long-term follow-up of patients.

METHODS

Patients

The number of patients who were diagnosed with chronic HCV infection between April 1998 and March 2007 in the Department of Hepatology, Toranomon Hospital, Tokyo, Japan was 5,400. Out of these, 890 were given antihypertensive therapy after confirmation of blood pressure $\geq$140 mm Hg systolic and/or $\geq$90 mm Hg diastolic on at least 3 visits and absence of secondary causes of hypertension, previous cardiovascular disease and stroke, and life threatening conditions. Blood pressure was measured by a physician with a mercury sphygmomanometer, with subjects sitting and relaxed for at least 10 min. Inclusion criteria were as follows: (1) antihypertensive therapy by losartan; (2) 45–75 years old; (3) no evidence of diabetes mellitus for 3 months before the initiation of anti-hypertensive therapy: a plasma glucose concentration of <126 mg per deciliter (6.9 mmol/L) in the fasting state, <200 mg per deciliter (11.0 mmol/L) in casual state and/or 2 hr after a 75-g oral glucose load; (4) features of chronic hepatitis or cirrhosis diagnosed by clinical features, laboratory tests, ultrasonographic findings, or histological findings; (5) positive for anti-HCV and HCV-RNA; (6) negative for hepatitis B surface antigens (HBsAg), antinuclear antibodies, or antimitochondrial antibodies in serum, as determined by radioimmunoassay or spot hybridization; (7) no evidence of HCC nodules as shown by ultrasonography and/or computed tomography; (8) no underlying systemic disease, such as systemic lupus erythmatosus, rheumatic arthritis. Patients with either of the following criteria were excluded from the study: (1) they were taking medicines known to alter glucose tolerance, (2) decompensated stage of cirrhosis with encephalopathy, icterus, or refractory ascites (3) they had illnesses that could seriously reduce their life expectancy or their ability to participate in the trial. Eighty patients were selected as losartan group. Patients were classified as having normal glucose group or pre-diabetes group base to the fasting plasma glucose (FPG), casual plasma glucose, or 2-hr plasma glucose: (1) normal glucose group was regarded as having FPG of <100 mg/dl, casual plasma glucose of <140 mg/dl, and/or 2-hr plasma glucose of <140 mg/dl, (2) pre-diabetes group was regarded as having FPG of 100–125 mg/dl, casual plasma glucose of 140–200 mg/dl, and/or 2-hr plasma glucose of 140–200 mg/dl [Genuth et al., 2003] The patients in the losartan-group received 50 mg of losartan orally once a day.

In the same period, 382 hypertensive patients with HCV positive chronic liver disease were treated with spironolactone. The 321 patients were applied with seven inclusion criteria and three exclusion criteria described in losartan group. One hundred sixty subjects in spironolactone group were selected from these 321 patients by matching 1:2 with losartan group for age and sex. Thus, differences of the cumulative appearance rate of T2DM in the losartan group and spironolactone group were compared. The patients in spironolactone group were treated with spironolactone at a dose of 25 or 50 mg once daily. Next, predictive factors for T2DM in both groups were assessed. The physicians in charge explained the purpose and method of antihypertensive treatment to each patient and/or patients' family, who gave their informed consent for the treatment. All of the studies were performed retrospectively by collecting and analyzing data from the patient records. This study had been approved by Institutional Review Board of Toranomon hospital.

Outcome Measures

The primary outcome was T2DM, diagnosed by the use of the 2003 criteria of the American Diabetes Association [Genuth et al., 2003]. That is, the criteria for the diagnosis of diabetes mellitus include: (a) casual plasma glucose $\geq$200 mg/dl; (b) FPG $\geq$126 mg/dl; (c) 2 hr post-glucose (oral glucose tolerance test) $\geq$200 mg/dl. At the same time, clinical records of cardiovascular events (angina pectoris, heart infarction) and stroke (cerebral infarction, cerebral bleeding) were examined.

Laboratory Inveastigation

Anti-HCV was detected using a second-generation enzyme-linked immunosorbent assay (ELISA II) (Abbott Laboratories, North Chicago, IL). HCV-RNA was determined by the Amplicor method (Cobas Amplicor HCV Monitor Test, v2.0, Roche, Tokyo, Japan). HBsAg was

tested by radioimmunoassay (Abbott Laboratories, Detroit, MI). The used serum samples were stored −80°C at the first consultation. Diagnosis of HCV infection was based on detection of serum HCV antibody and positive RNA. Height and weight were recorded at baseline and the body mass index (BMI) was calculated as weight (in kg)/height (in m^2)

Evaluation of Liver Cirrhosis and Fatty Liver

Status of liver cirrhosis was mainly determined on the basis of peritoneoscopy and/or liver biopsy. The 183 out of 260 were diagnosed by peritoneoscopy and/or liver biopsy. Liver biopsy specimens were obtained using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo, Japan), fixed in 10% formalin, and stained with hematoxylin-eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. The size of specimens for examination was more than six portal areas. Baseline liver histology of chronic hepatitis was classified according to the extent of fibrosis, into four stages in progression order: stage 1, periportal expansion; stage 2, portoportal septa; stage 3, portocentral linkage or bridging fibrosis; stage 4, liver cirrhosis [Desmet et al., 1994]. Remaining patients were diagnosed by clinical features, laboratory tests, and ultrasonographic findings.

Diagnosis of fatty liver was based on the presence of an ultrasonographic pattern consistent with bright liver (brightness and posterior attenuation) with stronger echoes in the hepatic parenchyma than in the renal or spleen parenchyma, vessel blurring, and narrowing of the lumen of the hepatic veins. US was performed with a high-resolution, real-time scanner (model SSD-2000; Aloka Co., Ltd, Tokyo Japan. Mode Logic-700 MR; GE-Yokokawa Medical Systems, Tokyo, Japan).

Follow-Up

The starting time of follow-up was the initiation of antihypertensive therapy. After that, patients were followed up monthly to tri-monthly in our hospital. Physical examination and biochemical tests were conducted at each examination together with regular check up. An overnight (12 hr) fasting blood sample or a casual blood sample was taken for routine analyses. These included transaminase activities, total cholesterol, platelet counts, and serum HCV RNA level. Twenty-one patients were lost to follow-up. Because the appearance of T2DM and death was not identified in these 21 patients, they considered as censored data in statistical analysis [Fleming et al., 1984]. Patients treated with antiviral agents were regarded as withdrawals at the time of starting the treatment of antiviral agents. Moreover, patients with change or addition of hypertensive drugs were regarded as withdrawals at the time of change or addition of hypertensive drugs. Finally, patients with decompensated stage of cirrhosis with encephalopathy, icterus, or refractory ascites were regarded as withdrawals.

Statistical Analysis

The cumulative appearance rate of T2DM was calculated from the initiation of hypertensive drugs using the Kaplan–Meier method. Differences in the development of T2DM were tested using the log rank test. Independent factors associated with the incidence rate of T2DM were analyzed by the Cox proportional hazard model. The following eleven variables were analyzed for potential covariates for incidence of T2DM after the time of initiation of hypertensive drugs at our hospital: age, sex, hepatic staging (chronic hepatitis or liver cirrhosis), BMI, glucose level, aspartate aminotransferase (AST), alanine aminotransferase (ALT) level, triglyceride level, total cholesterol level, and treatment. A P-value of <0.05 was considered significant. Data analysis was performed using the computer program SPSS package (SPSS 11.5 for Windows, SPSS, Chicago, IL).

RESULTS

Patients' Characteristics

Table I shows the characteristics of the 240 HCV positive and hypertensive patients enrolled in the present study. There were no significant differences in clinical profiles between the losartan and spironolactone group. The mean observation period was 5.2 years in losartan group and 5.4 years in spironolactone group. On side effects, two patients treated with losartan had episodes of dizziness. In spironolactone group, four patients had gynecomastia and two patients had dizziness. However, they could continue without stopping the antihypertensive therapy using losartan or spironolactone.

Incidence of T2DM in Hypertensive Patients With HCV

A total of 25 subjects (15 men and 10 women) developed T2DM during the observation period. Three patients in losartan group and 22 in spironolactone group developed T2DM. The 5th year cumulative appearance rates of T2DM were 5.9% in losartan group and 14.0% in spironolactone group (Fig. 1). Multivariate cox proportional hazards analysis showed that development of T2DM when anti-hypertensive drug was spironolactone (hazard ratio: 6.10; 95% confidence interval = 1.78–20.84; $P = 0.004$), histological staging was advanced (hazard ratio: 4.31; 95% confidence interval = 1.94–9.60; $P < 0.001$), fatty liver was present (hazard ratio: 3.28; 95% confidence interval = 1.47–7.27; $P = 0.004$), and patient had pre-diabetes (hazard ratio: 2.47; 95% confidence interval = 1.08–5.63; $P = 0.032$) (Table II). Our results indicate losartan causes about 60% reduction of the risk of T2DM development compared to spironolactone.

Figure 2 shows the impact of reduction due to administration of losartan on the incidence of T2DM in patients with liver cirrhosis, or pre-diabetes, or fatty liver. When patients with liver cirrhosis are treated with

TABLE I. Clinical Characteristics at the Time of Initiation of Anti-Hypertensive Drug

	Total	Losartan group	Spironolactone group	*P*-value
N	240	80	160	
Age (years)	65.2 ± 8.2	65.2 ± 8.0	65.2 ± 8.2	1.0
Sex (male/female)	120/120	40/40	80/80	1.0
Blood pressure				
Systolic (mm Hg)	161.8 ± 13.0	163.0 ± 14.1	160.9 ± 12.3	0.366
Diastolic (mm Hg)	94.3 ± 7.4	95.1 ± 8.2	93.9 ± 6.9	0.596
Staging (chronic hepatitis/liver cirrhosis)	194/46	64/16	130/30	0.863
F1/F2/F3/F4[a]	51/79/22/40	14/31/7/14	37/48/15/24	0.251
Fatty liver (+/−)[b]	48/192	14/66	34/126	0.608
BMI	23.7 ± 4.5	23.2 ± 3.5	23.9 ± 5.2	0.250
AST (IU/L)	77.5 ± 60.3	73.7 ± 49.2	78.8 ± 63.2	0.297
ALT (IU/L)	108.6 ± 99.8	108.8 ± 101.0	106.7 ± 94.2	0.604
Albumin (g/dl)	4.2 ± 0.4	4.2 ± 0.4	4.2 ± 0.5	0.717
γ-GTP (IU/L)	59.3 ± 58.5	58.2 ± 59.3	59.6 ± 60.8	0.862
Platelet count ($\times 10^4/mm^3$)	16.9 ± 5.6	15.8 ± 6.3	17.2 ± 5.4	0.089
Glucose level (prediabetes/normal)	42/198	15/65	27/133	0.722
T cholesterol (mg/dl)	172.8 ± 33.4	176.2 ± 53.5	172.5 ± 32.5	0.965
Triglyceride (mg/dl)	104.5 ± 47.1	97.0 ± 28.9	105.2 ± 48.9	0.063

Data are number of patients or mean ± standard deviation, ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; γ-GTP, γ-glutamyl transpeptidase.
[a]Histological diagnosis of the liver.
[b]Diagnosis of fatty liver by the ultrasonography.

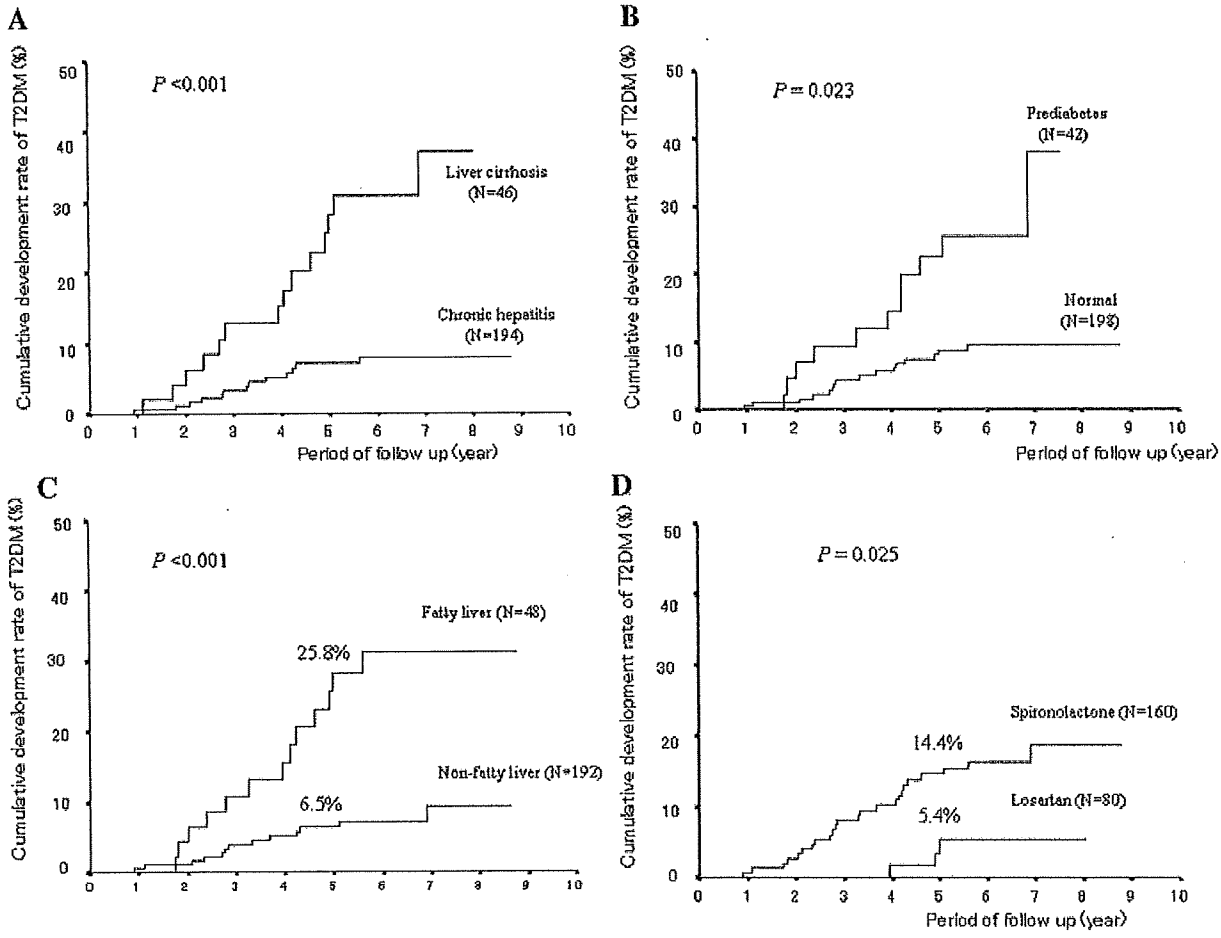

Fig. 1. Cumulative development rate of T2DM in patients treated with interferon. **Panel A**: Cumulative development rate of T2DM based on difference of hepatic fibrosis; (**Panel B**), cumulative development rate of T2DM based on the difference of glucose level; (**Panel C**), cumulative development rate of T2DM based on the difference of fatty liver; (**Panel D**), cumulative development rate of T2DM based on the difference of anti-hypertensive drugs.

TABLE II. Predictive Factors for T2DM Development

Variables	N	Univariate analysis		Cox-regression	
		HR (95% CI)	P	HR (95% CI)	P
Age (years, ≥65/<65)	121/119	2.28 (1.02–5.07)	0.044		
Sex (female/male)	120/120	0.60 (0.28–1.28)	0.184		
BMI (≥25/<25)	60/180	2.42 (1.091–5.33)	0.028		
Maximum BMI (≥25/<25)	55/141	1.76 (0.76–4.06)	0.190		
Fatty liver (+/−)	48/192	4.35 (2.01–5.07)	<0.001	3.28 (1.47–7.27)	0.004
Genotype (1/2)	162/45	0.91 (0.39–2.88)	0.905		
ALT (IU/L, ≥50/<50)	151/89	1.14 (0.38–3.42)	0.822		
Glucose level (prediabetes/normal)	42/198	2.93 (1.33–6.48)	0.022	2.47 (1.08–5.63)	0.032
Triglyceride (mg/dl, ≥150/<150)	34/135	1.85 (0.83–5.98)	0.095		
Cholesterol (mg/dl, <220/≥220)	172/40	0.54 (0.06–5.16)	0.590		
Staging (liver cirrhosis/chronic hepatitis)	46/194	4.25 (1.97–9.18)	0.023	4.31 (1.94–9.60)	<0.001
AST (IU/L, ≥38/<38)	168/72	0.96 (0.32–2.881)	0.942		
Treatment (spironolactone/losartan)	160/80	3.94 (1.19–13.15)	0.025	6.10 (1.78–20.84)	0.004

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; HCV, hepatitis C virus; HR, hazards ratio.

losartan, losartan could statistically reduce the onset of T2DM compared to those with spironolactone.

Incidence of Cardio Vascular Disease or Stroke in Patients With HCV

A total of eight subjects (five men and three women) developed vascular events during the observation period. In losartan group, two patients developed stroke. In spironolactone group, three patients developed cardiovascular disease and another three patients developed stroke.

Figure 3 shows the impact of reduction due to difference of antihypertensive drugs on the incidence of cardiovascular disease or stroke in two groups. There was little difference on losartan group and spironolactone group.

DISCUSSION

We have described the development incidence of T2DM after the initiation of antihypertensive therapy in HCV positive patients treated with antihypertensive drugs in the present study. The present study was limited by a retrospective cohort trial. About the sample size in losartan and spironolactone group, the number of the patients enrolled in the present study was sufficient to detect hazards ratios of about threefold with 80% power at the 5% level of significance. The strength of the present study is a long-term follow-up in the patients included.

The present study shows several findings with regard to development of T2DM after the initiation of losartan or spironolactone for HCV positive and hypertensive patients. First, the T2DM development rate in losartan group was lower than that in spironolactone group. The administration of losartan caused about 60% reduction in the onset of T2DM in the course of follow-up. What losartan enhances the insulin sensitivity has been reported by some authors [Iimura et al., 1995; Ando and Fujita, 2006; Alderman, 2008]. However, protection of T2DM development by losartan in the present study was effective compared to that based on Dahlöf's report [Dahlöf et al., 2002]. This discrepancy is thought to be due to difference of race and HCV infection. Our previous study shows that clearance of HCV causes a two-thirds reduction of the onset of T2DM in hepatitis C virus positive patients treated with interferon [Arase et al., 2009]. This means that patients with HCV have a high tendency of the onset of T2DM. Moreover, although the prevalence of T2DM is increasing dramatically in USA, increases in newly developed and developing countries in Asia have been ever greater [Yoon et al., 2007]. Thus, Asian patients with HCV are thought to have high risk of T2DM. Anti-diabetic effect of losartan may also enhance in patients with high risk of T2DM.

Though the role of losartan in preventing development of DM remains speculative, the following possible mechanism have been reported, (1) losartan elevates the serum level of adiponectin that improves insulin sensitivity [Clasen et al., 2005]; (2) losartan enhance the insulin-like growth factor (IGF)-1 that plays a protective role in the development of glucose intolerance [Zandbergen et al., 2006].

Second, in addition to administration of spironolactone, the present study suggests that aging, progression of hepatic staging, pre-diabetes enhanced the onset of T2DM in HCV patients treated with anthypertensive drugs.

The present study indicates that losartan reduce the onset of T2DM in Japanese patients with HCV. Our retrospective study suggests that the annual incidence of T2DM among patients with HCV was determined to be 1.0–1.1% in losartan group and 2.8–3.0% in spironolactone group. Moreover, several lines of evidence have shown that angiotensin receptor antagonist can have a beneficial role in the early stages of hepatic fibrosis of patients with hepatitis C [Terui et al., 2002]. Thus, when physicians regarding the daily management of patients with virus hepatitis give antihypertensive therapy for HCV patients, they should

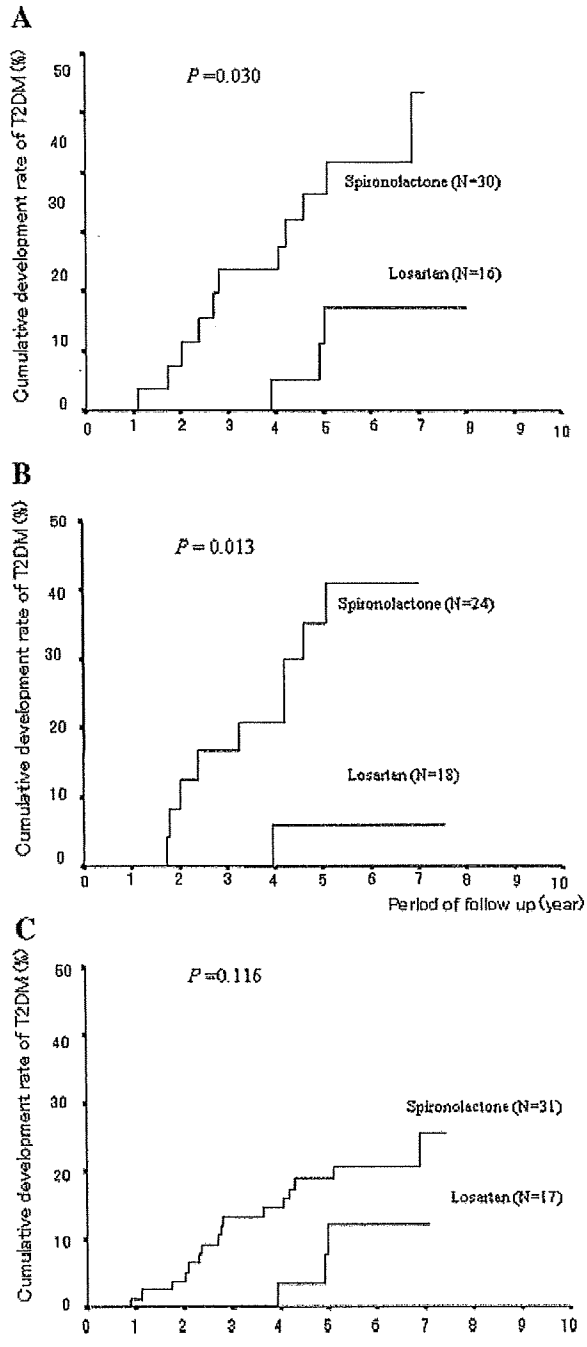

Fig. 2. Cumulative development rate of T2DM in patients with losartan or spironolactone. **Panel A**: Cumulative development rate of T2DM based on the difference of anti-hypertensive drugs in patients with liver cirrhosis; (**Panel B**), cumulative development rate of T2DM based on the difference of anti-hypertensive drugs in patients with pre-diabetes; (**Panel C**), cumulative development rate of T2DM based on the difference of anti-hypertensive drugs in patients with fatty liver.

consider the indication of losartan for protecting the onset of T2DM and progression of liver fibrosis.

In conclusion, our results indicate losartan causes about 60% reduction of the risk of T2DM development in HCV positive, hypertensive, Japanese patients.

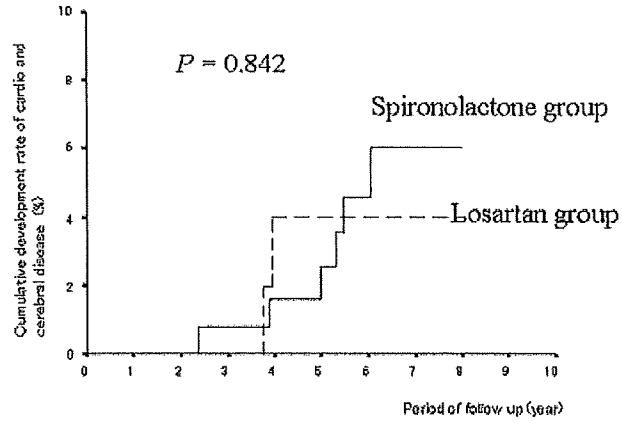

Fig. 3. Cumulative development rate of cardiovascular disease and stroke based on the difference of anti-hypertensive drug.

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Virological and Biochemical Features in Elderly HCV Patients with Hepatocellular Carcinoma: Amino Acid Substitutions in HCV Core Region as Predictor of Mortality after First Treatment

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Key Words
Hepatitis C virus · Core region · Hepatocellular carcinoma · Mortality

Abstract
Aims: We showed previously that amino acid (aa) substitutions in HCV genotype 1b (HCV-1b) core region are negative predictors of virological response to peginterferon + ribavirin therapy, and also risk factors of hepatocarcinogenesis. The aim of this study was to evaluate the impact of core aa substitutions on mortality in elderly patients. ***Methods:*** We compared the characteristics and survival of 92 elderly (≥75 years) patients with HCV-related hepatocellular carcinoma (HCC) (including 62 patients with HCV-1b) with those of 44 younger patients (<50 years, 34 patients with HCV-1b). ***Results:*** For all patients, univariate analysis identified female sex, history of blood transfusion, preserved liver function and glucose metabolism as significant variables in the elderly patients. In patients with HCV-1b-related HCC, univariate analysis identified preserved lipid metabolism as significant variable in addition to significant variables in overall patients. In elderly patients with HCV-1b-related HCC, multivariate analysis identified male sex, methionine of core aa91, and non-radical therapy as factors that influenced mortality after first treatment for HCC. ***Conclusions:*** Our results characterized elderly patients who develop HCC after HCV-1b infection, and suggested that aa substitutions of HCV-1b core region correlate with mortality of patients after first treatment for HCC.

Introduction

Hepatitis C virus (HCV) usually causes infection, which can result in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [1–6]. In patients with HCV-chronic hepatitis, treatment with interferon (IFN) can induce viral clearance and marked biochemical and histological improvement [7, 8].

In Japan, the number of elderly HCC patients with HCV infection has increased in recent years, especially the proportion of female patients with chronic hepatitis [9–13]. On the other hand, Saneto et al. [12] reported that radical therapy for elderly HCC patients with HCV infection improved their survival rate. However, the impact of virological factors on the clinical features and survival

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rate after treatment of elderly patients with HCC is not clear at present.

Previous studies indicated that amino acid (aa) substitutions at position 70 and/or 91 in the HCV core region of genotype 1b are predictors of poor virological response to peginterferon + ribavirin therapy [14–20], and also risk factors for hepatocarcinogenesis [21, 22]. However, it is not clear at this stage whether aa substitutions in the core region of genotype 1b influence the clinical features and survival rate after treatment of HCC patients.

The study subjects were 136 consecutive patients infected with HCV, including 96 patients infected with HCV-1b. The aims of the study were the following: (1) to compare the clinicopathological features and virological factors of elderly patients (≥75 years old) with HCV infection, with those of young patients (≤50 years old), with a special emphasis on those with HCV-1b, which is the dominant genotype in Japan, and (2) to analyze the predictive factors, especially virological factors, associated with mortality in HCV-1b patients who received first therapy for HCC.

Methods

Patients

From January 1980 to December 2005, 1,804 HCC patients were treated at Toranomon Hospital, Tokyo, Japan. HCV antibody-positive and hepatitis B surface antigen-negative patients were included in this study. Of these consecutive patients, 92 (5.1%) were ≥75 years old, while 44 (2.4%) were ≤50 at the time of development of primary HCC. We defined those aged ≥75 years as the 'elderly' group and those aged ≤50 as the 'young' group. The HCV genotype 1b-related HCC group (n = 96) comprised 62 elderly patients and 34 young patients. The study protocol was approved by the Human Ethics Review Committee of Toranomon Hospital.

We reviewed the medical records of the patients and compared their clinical characteristics and laboratory data. Sex, HCV genotype, history of IFN therapy, history of blood transfusion, alcohol consumption, presence of diabetes mellitus (only patients who needed treatment with oral antidiabetic agents or insulin injections), body weight, body mass index (BMI), family history of liver disease (i.e., chronic hepatitis, liver cirrhosis or HCC, whether or not with HCV infection) were recorded. In this study, diabetes mellitus was diagnosed based on treatment of diabetes rather than by analysis of HbA1c data.

To distinguish chronic hepatitis from cirrhosis, we used the discriminate score reported previously by our institution [23]. In brief, the score was generated by stepwise selection of 20 variables from among 168 peritoneoscopy- and biopsy-proven patients with chronic hepatitis and 37 patients with cirrhosis who were infected with HCV. It is calculated as follows: $z = [0.124 \times \gamma\text{-globulin (\%)}] + [0.001 \times \text{hyaluronic acid (}\mu\text{g/l)}] - [0.075 \times \text{platelet count (}\times 10^4/\mu\text{l)}] - [0.413 \times \text{sex (male, 1; female, 2)}] - 2.005$. A positive z value denotes cirrhosis and a negative value indicates chronic hepatitis.

Laboratory Data

For laboratory data, we recorded platelet count, prothrombin activity, total bilirubin (T-Bil), alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, α-fetoprotein (AFP), the retention rate of indocyanine green dye at 15 min (ICG R15), hyaluronic acid, total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), fasting glucose (FBG), serum insulin, serum iron, ferritin and levels of HCV RNA (TaqMan HCV PCR [24]).

Nucleotide Sequencing of HCV-1b Core and NS5A Gene

With the use of HCV-J (accession No. D90208) as a reference [25], the sequence of 1–191 aa in the core protein of genotype 1b was determined and then compared with the consensus sequence constructed from 50 clinical samples to detect substitutions at aa70 of arginine (Arg70) or glutamine/histidine (Gln70/His70) and aa91 of leucine (Leu91) or methionine (Met91) [14]. The sequence of 2209–2248 aa in the NS5A of genotype 1b (IFN-sensitivity determining region [ISDR]) reported by Enomoto et al. [26, 27] was also determined, and the numbers of aa substitutions in ISDR were defined as wild-type (≤1) or mutant type (≥2).

The aa substitutions of the core region and NS5A-ISDR were analyzed by direct sequencing [14, 26, 27]. HCV RNA was extracted from serum samples at the start of treatment and reverse transcribed with random primer and MMLV reverse transcriptase (Takara Syuzo, Tokyo). Nucleic acids were amplified by PCR using the following primers: (a) Nucleotide sequences of the core region: the first-round PCR was performed with CC11 (sense, 5′-GCC ATA GTG GTC TGC GGA AC-3′) and e14 (antisense, 5′-GGA GCA GTC CTT CGT GAC ATG-3′) primers, and the second-round PCR with CC9 (sense, 5′-GCT AGC CGA GTA GTG TT-3′) and e14 (antisense) primers. (b) Nucleotide sequences of NS5A-ISDR: the first-round PCR was performed with ISDR1 (sense, 5′-ATG CCC ATG CCA GGT TCC AG-3′) and ISDR2 (antisense, 5′-AGC TCC GCC AAG GCA GAA GA-3′) primers, and the second-round PCR with ISDR3 (sense, 5′-ACC GGA TGT GGC AGT GCT CA-3′) and ISDR4 (antisense, 5′-GTA ATC CGG GCG TGC CCA TA-3′) primers ([a] hemi-nested PCR; [b] nested PCR). All samples were initially denatured at 95° for 15 min. The 35 cycles of amplification were set as follows: denaturation for 1 min at 94°, annealing of primers for 2 min at 55°, and extension for 3 min at 72° with an additional 7 min for extension. Then 1 μl of the first PCR product was transferred to the second PCR reaction. Other conditions for the second PCR were the same as the first PCR, except that the second PCR primers were used instead of the first PCR primers. The amplified PCR products were purified by the QIA quick PCR purification kit (Qiagen, Tokyo) after agarose gel electrophoresis and then used for direct sequencing. Dideoxynucleotide termination sequencing was performed with the Big Dye Deoxy Terminator Cycle Sequencing kit (PerkinElmer, Tokyo).

Hepatocellular Carcinoma

The diagnosis of HCC was based on imaging studies and histopathological examination. However, when a typical hypervascular staining pattern was obtained on angiography or a hyperattenuating nodule was detected on the arterial phase of dynamic

computed tomography, the nodule was considered HCC without histopathological examination. On the other hand, when the hepatic nodule did not show the aforementioned patterns, a fine-needle biopsy was performed to exclude or diagnose HCC. Tumor numbers, size, tumor marker levels (AFP), the first therapy method for HCC, recurrence of HCC after the first therapy, and the survival rate were also recorded.

Statistical Analysis

Non-parametric tests (χ^2 test and Mann-Whitney U-test) were used to compare the characteristics of these groups. The cumulative survival rate was calculated using the Kaplan-Meier technique, differences between the survival curves were tested using the log-rank test. Statistical analyses of survival according to specific variables were calculated period from the first treatment for HCC. Stepwise Cox regression analysis was used to determine independent predictive factors that were associated with mortality. We also calculated the odds ratios and 95% confidence intervals (95% CI). The potential factors associated with mortality included the following variables: sex, family history of liver disease, history of blood transfusion, history of IFN therapy, alcohol consumption (total amount of ethanol consumption by the time of HCC diagnosis, ≥500 kg), diabetes mellitus under treatment, body weight, BMI, prothrombin activity (PT), platelet counts, serum albumin, total bilirubin (T-Bil), aspartate aminotransferase (AST), alanine aminotransferase (ALT), ICG R15, TC, HDL-C, (LDL-C, TG, serum iron, ferritin, fasting FBG, insulin, HOMA-IR, levels of HCV RNA, AFP, aa substitution in the core and ISDR of NS5A. We considered variables with $p < 0.05$ as significant and those with $p < 0.10$ as marginally significant. Statistical analyses were performed using the SPSS software (SPSS, Inc., Chicago, Ill., USA).

Results

Patient Characteristics with HCV Infection

Table 1 shows the clinical characteristics and laboratory data of the young and elderly groups with HCV-related HCC. Among the 92 elderly patients, 53% were men and 47% were women. For the younger group, 89% were men and 11% were women. The proportion of female patients was significantly higher in the elderly group than in the young group ($p < 0.001$). The proportion of patients with HCV genotype 1b was not different between two age groups ($p = 0.366$). Patients with family history of liver disease were more common in the young group than the elderly group (30.4 vs. 5.4%, $p < 0.001$). A larger proportion of elderly patients had history of blood transfusion than the younger group (32.6 vs. 52.2%, $p < 0.001$). On the other hand, a larger proportion of younger patients were alcohol drinkers than in the elderly group (23.9 vs. 10.9%, $p = 0.044$). Most of the elderly patients had not been treated with IFN before the diagnosis of HCC (37.0 vs. 6.5%, $p < 0.001$). Body weight and BMI were lower in the elderly group than in the younger group ($p < 0.001$ in both variables). In the elderly group, PT was higher than in the younger group ($p < 0.001$), whereas T-Bil and ALT were lower in the elderly group ($p < 0.001$ and $p = 0.035$, respectively). Serum insulin level and HOMA-IR were lower in the elderly group than in the younger group ($p = 0.029$ and $p = 0.032$, respectively). There were no significant differences between two groups in other variables.

Patient Characteristics with HCV-1b Infection

Table 2 shows the clinical characteristics and laboratory data of the young and elderly HCC groups with HCV-1b infection. Men formed 53% and women 47% of the 62 elderly patients, while in the younger group, these were 91 and 9%, respectively. The proportion of female patients was significantly higher in the elderly group than in the young group ($p < 0.001$). Patients with family history of liver disease were more common in the young than elderly group (29.4 vs. 5.4%, $p < 0.001$). More elderly patients had a history of blood transfusion than the younger group (51.6 vs. 26.5%, $p < 0.016$). However, the proportion of alcohol drinkers was higher for the younger group than elderly group (20.6 vs. 4.8%, $p = 0.029$). A higher proportion of elderly patients had not been treated with IFN before the diagnosis of HCC (41.2 vs. 8.1%, $p < 0.001$). Body weight and BMI were lower in the elderly group than in the younger group ($p < 0.001$ in both variables). In the elderly group, PT was higher than in the younger group ($p = 0.001$), whereas T-Bil and ALT was lower in the elderly group ($p < 0.001$ and $p = 0.006$, respectively). Serum insulin level and HOMA-IR were lower in the elderly than in the younger group ($p = 0.034$ and $p = 0.023$, respectively). HDL-C level was higher in the HCV-1b infection-HCC elderly group than the younger group. There were no significant differences in other factors between the two groups.

Survival According to aa Substitutions of HCV-1b Core Region

We analyzed the survival rates after the first treatment for HCC, according to substitutions in core aa70 and aa91, using the Kaplan-Meier technique for elderly and young patients with HCV-1b infection-HCC. In the elderly patients, the cumulative survival rate according to substitutions of core aa70 was not different (log-rank test; $p = 0.821$, fig. 1a). For Arg70 and Gln70/His70, the cumulative survival rates were 46, 43% at the end of 3 years; 38, and 17% at the end of 5 years, respectively. However, for the core aa91, the cumulative survival rates varied significantly according to the type of substitution (log-rank test; $p = 0.009$, fig. 1b). For Leu91 and Met91, the cumula-

Table 1. Profile of elderly and young HCC patients with HCV infection

Variable	Young group (n = 46)	Elderly group (n = 92)	p value
Male/female	41/5	49/43	<0.001
Family history of liver disease	30.4% (14/46)	5.4% (5/92)	<0.001
History of blood transfusion	32.6% (15/46)	52.2% (48/92)	0.026
History of interferon therapy	37.0% (17/46)	6.5% (6/92)	<0.001
Alcohol consumption (>500 kg)	23.9% (11/46)	10.9% (10/92)	0.044
Diabetes mellitus (under treatment)	17.4% (8/46)	6.3% (13/92)	0.615
Discriminate score (>0/≤0/ND)	27/12/7	43/42/7	0.077
Body weight, kg[a]	68.5 (51.8–87.2)	51.7 (35.5–83.8)	<0.001
Body mass index[a]	23.8 (19.1–31.3)	22.2 (14.8–29.1)	<0.001
Prothrombin activity, %[a]	75 (13.4–100)	88.8 (49.9–125.4)	<0.001
Platelet count, $\times 10^4/\mu l$[a]	10.6 (2.8–25.7)	11.3 (3.2–31.7)	0.173
Serum albumin, g/dl[a]	3.5 (2.1–4.3)	3.5 (2.3–4.5)	0.776
T-Bil, mg/dl[a]	1.2 (0.4–6.4)	0.9 (0.5–2.9)	<0.001
AST, IU/l[a]	61 (22–311)	57 (20–138)	0.683
ALT, IU/l[a]	55 (10–425)	46 (10–162)	0.035
ICG R15, %[a]	32 (6–68)	30 (8–86)	0.458
Total cholesterol, mg/dl[a]	153 (85–207)	158 (91–233)	0.209
HDL-C, mg/dl[a]	41 (16–68)	42 (20–89)	0.137
LDL-C, mg/dl[a]	80 (38–141)	94 (52–165)	0.123
Triglyceride, mg/dl[a]	87 (38–538)	81 (33–254)	0.248
Serum iron, μg/dl[a]	169 (7–326)	135 (13–513)	0.169
Ferritin, μg/l[a]	325 (10–1,271)	89 (10–726)	0.063
Fasting blood glucose, mg/dl[a]	97 (39–313)	95 (68–159)	0.970
Insulin, μU/ml[a]	12.9 (3.6–71.2)	11.2 (1.9–69.9)	0.029
HOMA-IR[a]	3.2 (0.7–29.2)	2.6 (0.3–37.2)	0.032
Level of HCV RNA, log IU/ml[a]	5.6 (3.5–7.6)	6.1 (3.1–7.3)	0.177
AFP, μg/l[a]	29 (2–2,700)	37 (2–16,300)	0.841
Number of HCC, solitary/multiple	27/19	58/34	0.709
Size of largest tumor, mm[a]	20 (80–170)	20 (7–72)	0.716

HCC = Hepatocellular carcinoma; T-Bil = total bilirubin; AST = aspartate aminotransferase; ALT = alanine aminotransferase; ICG R15 = retention rate of indocyanine green dye at 15 min; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; AFP = α-fetoprotein; HOMA-IR = homeostasis model assessment of insulin resistance.

[a] Median (range).

tive survival rates were 57, 18% at the end of 3 years, and 41, 0% at the end of 5 years, respectively.

In the young patients, the cumulative survival rates were significantly different according to the type of substitution of core aa70 (log-rank test; p = 0.012, fig. 2a). For Arg70 and Gln70/His70, the cumulative survival rates were 82, 65% at the end of 3 years, and 60, 22% at the end of 5 years, respectively. For the core aa91, the cumulative survival rates according to the type of substitution were significantly different (log-rank test; p = 0.026, fig. 2b). For Leu91 and Met91, the cumulative survival rates were 87, 54% at the end of 3 years, and 60, 22% at the end of 5 years, respectively.

Multivariate Analysis of Factors Associated with Mortality after First Treatment of HCC

We then analyzed the data to determine those variables that influenced mortality after first treatment of HCC. Univariate analysis showed a relationship between each of the following six parameters and mortality in the elderly group: sex (male, p = 0.0287), treatment (non-radical therapy, p = 0.0203), PT (<80%, p = 0.0099), tumor size (≥30 mm, p = 0.0477), substitution of core aa91 (Met91, p = 0.0091) and serum iron level (≥121 μg/dl, p = 0.0526). These factors were entered into multivariate analysis, which then identified three independent parameters that tended to or significantly influenced mor-

Table 2. Profile of elderly and young HCC patients with HCV genotype 1b infection

	Young group (n = 34)	Elderly group (n = 62)	p value
Male/female	31/3	33/29	<0.001
Family history of liver disease	29.4% (10/34)	5.4% (3/62)	<0.001
History of blood transfusion	26.5% (9/34)	51.6% (32/62)	0.016
History of interferon therapy	41.2% (14/34)	8.1% (5/62)	<0.001
Alcohol consumption (≥500 kg)	20.6% (7/34)	4.8% (3/62)	0.029
Diabetes mellitus (under treatment)	20.6% (7/34)	14.5% (9/62)	0.400
Body weight, kg[a]	68.9 (51.8–87.2)	51.2 (35.5–83.8)	<0.001
Body mass index[a]	24.3 (19.1–31.3)	22.2 (14.8–29.1)	<0.001
Prothrombin activity, %[a]	78.8 (13.4–100)	90.1 (52.0–125.4)	0.001
Platelet count, $\times 10^4/\mu l$[a]	10.9 (2.8–25.7)	11.3 (3.2–31.7)	0.117
Serum albumin, g/dl[a]	3.4 (2.1–4.3)	3.5 (2.3–4.5)	0.199
T-Bil, mg/dl[a]	1.2 (0.4–6.4)	0.9 (0.5–2.9)	<0.001
AST, IU/l[a]	65 (22–229)	58 (20–138)	0.210
ALT, IU/l[a]	65 (10–295)	48 (11–106)	0.006
ICG R15, %[a]	32 (6–65)	32 (12–86)	0.410
Total cholesterol, mg/dl[a]	153 (85–197)	158 (91–233)	0.174
HDL-C, mg/dl[a]	38 (3–68)	44 (20–89)	0.010
LDL-C, mg/dl[a]	86 (41–122)	94 (52–165)	0.185
Triglyceride, mg/dl[a]	93 (38–538)	79 (33–223)	0.243
Serum iron, μg/dl[a]	123 (7–326)	138 (13–405)	0.655
Ferritin, μg/l[a]	140 (10–1,271)	88 (10–726)	0.323
Fasting blood glucose, mg/dl[a]	96 (56–313)	95 (68–159)	0.741
Insulin, μU/ml[a]	13.7 (3.6–204.0)	10.6 (1.9–102.0)	0.034
HOMA-IR[a]	3.7 (0.7–85.7)	2.6 (0.3–19.4)	0.023
Substitution of core amino acid 70 (Arg/Gln or His/ND)	17/11/6	24/18/20	0.809
Substitution of core amino acid 91 (Leu/Met/ND)	13/15/6	27/14/20	0.457
Mutation of NA5A-ISDR (≤1/>2/ND)	21/6/7	16/24/22	0.186
Level of HCV RNA, log IU/ml[a]	5.9 (3.5–7.6)	6.3 (3.4–7.3)	0.220
AFP, μg/l[a]	25 (2–1,690)	34 (2–1,150)	0.806
Number of HCC, solitary/multiple	14/20	21/41	0.293
Size of largest tumor, mm[a]	18 (8–170)	20 (7–72)	0.502

HCC = Hepatocellular carcinoma; T-Bil = total bilirubin; AST = aspartate aminotransferase; ALT = alanine aminotransferase; ICG R15 = retention rate of indocyanine green dye at 15 min; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; AFP = α-fetoprotein; HOMA-IR = homeostasis model assessment of insulin resistance; Arg = arginine; Gln = glutamine; His = histidine; Leu = leucine; Met = methionine.

[a] Median (range).

tality: sex (male, p = 0.021), substitution of core aa91 (Met91, p = 0.012), and treatment (non-radical therapy, p = 0.085) (table 3).

Similar analyses were conducted in the young group. Univariate analysis showed a relationship between each of the following parameters and mortality in the young group: first therapy (non-radical therapy, p = 0.0113), AFP (≥21 μg/l, p = 0.0329), AST (<57 IU/l, p = 0.0813), ALT (<51 IU/l, p = 0.0275), tumor number (solitary, p = 0.0048), presence of diabetes mellitus (p = 0.0001), FBG (≥111 mg/dl, p = 0.0217) and substitution of aa in core region 70 and 91 (Gln70/His70 and Met91, p = 0.0122 and p = 0.0258, respectively). These factors were entered into multivariate analysis, which then identified six independent parameters that tended to or significantly influenced mortality: treatment (non-radical therapy, p = 0.002), ALT (<51 IU/l, p = 0.045), AFP (≥21 μg/l, p = 0.021), FBG (≥111 mg/dl, p = 0.019), substitution of core aa91 (methionine, p = 0.011) and tumor number (solitary, p = 0.084) (table 4).