

Figure 1. Cumulative survival rate of HCC patients according to chronic viral hepatitis infection.

still diagnosed at an advanced stage and their survival time is therefore short. Patients with chronic HBV and/or HCV infection in addition to cirrhosis should be monitored with USG and/or CT and/or MRI of the liver to detect tumors at an early stage. HCC surveillance using imaging modalities is usually performed at 6-month intervals [28,29]. There is a large population of individuals infected with HCV, HBV, or both in whom cancer is in the latency period. For those who harbor chronic HCV and/or HBV infections, attention must be focused on the detection of HCC at an early stage. In this study, more than 75% of patients with HCC were positive for HBV and/or HCV. Additionally, our data showed patients with HCC-nonBC to generally be diagnosed at an advanced stage. Thus, the target population for HCC surveillance must be easily identifiable. However, it will not be easy to select appropriate subjects for screening of HCC among those negative for both HBsAg and HCVAb.

AFP has long been considered the ideal serological marker for detecting HCC. Persistently elevated AFP is well known to be related to the presence of HCC and its determination can facilitate better identification of patients at risk. However, in our present dataset, the median serum AFP level in HCC-nonBC was not abnormal, whereas those in HCC-B and HCC-C cases were abnormal. Few early-stage HCC-nonBC cases present with abnormal AFP serum levels. Several reports have shown elevated AFP to be a risk factor for HCC development in HCV and/or HBV patients [24,30–36]. However, our results suggest AFP alone to be insufficient for HCC-nonBC surveillance.

Since Liebman et al. demonstrated DCP to be a useful marker for HCC diagnosis, many studies have compared DCP and AFP. Several investigations have made comparisons of the usefulness of DCP and AFP for HCC diagnosis [37–40]. However, whether AFP is superior to DCP in all cases is still controversial. Even the sensitivities and specificities reported by these studies were quite different. One reason for these differences involves the use of different cut-off

Table 2. Univariate analysis of factors associated with HCC-nonBC.

Parameters	Hazard ratio	P value
Age (years) ≥70	1.59	<0.001
Sex Female	0.67	<0.001
BMI (kg/m ²) ≥25	1.85	<0.001
Alcohol consumption		
None	1	–
Not excessive	2.57	<0.001
Excessive	12.41	<0.001
Diabetes mellitus (%)		
+	2.96	<0.001
Underlying liver disease		
Normal	1	–
Chronic hepatitis	0.20	<0.001
Cirrhosis	0.20	<0.001
Child-Pugh grade		
A	1	–
B	0.92	0.446
C	1.34	0.131
Platelets (10 ³ /μL) ≥116	2.22	<0.001
AST (IU/L) <56	2.15	<0.001
ALT (IU/L) <46	2.64	<0.001
PT (%) ≥83	1.26	0.016
Bil (mg/dL) <0.9	0.89	0.229
Alb (mg/dL) ≥3.7	1.06	0.547
AFP (ng/mL)		
<20	1	–
20–199	0.48	<0.001
≥200	0.82	0.079
DCP (mAU/mL)		
<40	1	–
40–199	1.66	<0.001
≥200	2.73	<0.001
TNM stage		
I	1	–
II	1.66	<0.001
III	2.73	<0.001
IV	2.22	<0.001

Table 3. Multivariate analysis of factors associated with HCC-nonBC.

Parameters	Hazard ratio	95% CI	P value
Age (years) ≥70	1.63	1.21–2.20	0.001
Sex Female	1.73	1.33–2.85	<0.001
BMI (kg/m ²) ≥25	2.12	1.58–2.83	<0.001
Alcohol consumption			
None	1	–	–
Not excessive	3.41	2.43–4.79	<0.001
Excessive	14.73	9.48–22.9	<0.001
Diabetes mellitus (%)			
(+)	2.42	1.82–3.22	<0.001
Underlying liver disease			
Normal	1	–	–
Chronic hepatitis	0.46	0.01–0.16	<0.001
Cirrhosis	0.52	0.02–0.19	<0.001
Platelets (10 ³ /μL) ≥116	1.88	1.35–2.60	<0.001
AST (IU/L) <56	1.47	1.01–2.10	0.411
ALT (IU/L) <46	2.08	1.47–2.94	<0.001
PT (%) ≥83	0.97	0.71–1.32	0.826
AFP (ng/mL)			
<20	1	–	–
20–199	0.60	0.42–0.85	0.005
≥200	0.63	0.43–0.92	0.079
DCP (mAU/mL)			
<40	1	–	–
40–199	1.64	1.13–2.39	0.010
≥200	1.88	1.35–2.60	0.018
TNM stage			
I	1	–	–
II	1.67	1.13–2.48	0.011
III	1.88	1.19–2.96	0.007
IV	2.40	1.32–4.35	<0.001

CI – confidence interval.

values in the various studies (e.g., 40, 60, and 100 mAU/mL for DCP; and 20, 100, and 200 ng/mL for AFP). Other possible reasons include differences in the causes of the underlying liver diseases, and patients with cirrhosis tending to have higher AFP levels than those with chronic hepatitis [36,41]. Another possible reason for these differences might be etiological differences in liver diseases among the

patients examined in prior studies. In this study, the median AFP level in HCC-nonBC was significantly lower than that in either HCC-B or HCC-C, whereas the median DCP level was significantly higher. Our data suggest that DCP levels differ among liver diseases with different etiologies. The high value identified in our study may be related to the higher DCP values in patients without hepatitis virus infection.

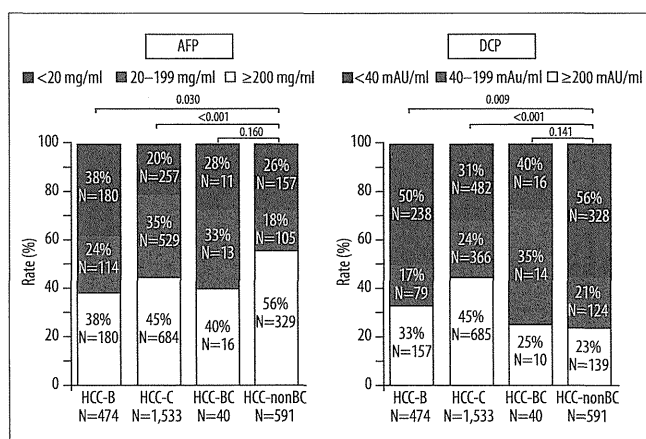


Figure 2. The positive rate of AFP (≥ 20 ng/ml) and DCP (≥ 40 mAU/ml) in HCC-B, HCC-C, HCC-BC and HCC-nonBC.

Table 4. Median AFP and DCP levels in HCC-B, HCC-C, HCC-BC and HCC-nonBC according to TNM stage.

	HCC-B	HCC-C	HCC-BC	HCC-nonBC
All patients				
Number	474	1,533	40	591
AFP (ng/mL) (range)	60 (1–2,920,000)*	25 (1–1,438,472)*	29 (3–189,850)***	13 (1–8,145,000)
DCP (mAU/mL) (range)	4,990 (4–1,497,560)	418 (1–871,700)*	612 (10–266,260)	3,077 (5–265,000,000)
TNM stage I				
Number	103	480	13	77
AFP (ng/mL) (range)	15 (1–6,300)**	16 (1–3,188)*	28 (3–2,120)*	6 (1–9,820)
DCP (mAU/mL) (range)	26 (3–1,038)**	25 (1–20,448)*	39 (14–353)	44 (12–12,224)
TNM stage II				
Number	150	624	11	254
AFP (ng/mL) (range)	14 (1–181,150)**	24 (1–200,000)*	17 (5–952)	8 (1–114,907)
DCP (mAU/mL) (range)	72 (4–233,780)*	53 (2–74,493)*	173 (10–831)	255 (5–369,000)
TNM stage III				
Number	92	319	12	157
AFP (ng/mL) (range)	77 (2–453,000)***	48 (1–196,000)**	47 (4–72,727)	25 (1–246,940)
DCP (mAU/mL) (range)	535 (10–172,000)	305 (4–14,410) ***	206 (16–4,039)	460 (10–109,350)
TNM stage IV				
Number	129	102	4	103
AFP (ng/mL) (range)	4,450 (1.5–2,920,000)**	2,379 (2–1,438,472)	64,838 (2882–189,850)***	914 (1–8,145,000)
DCP (mAU/mL) (range)	9,573 (10–1,497,560)	8,954 (19–871,700)	104,254 (755–266,260)	3,340 (12–265,000,000)

* $p \leq 0.001$ vs. HCC-nonBC; ** $p \leq 0.01$ vs. HCC-nonBC; *** $p \leq 0.05$ vs. HCC-nonBC.

The biological function of AFP is still not well identified. Since AFP is similar to albumin, it is possible that AFP function as a carrier for several ligands such as bilirubin, fatty acids, steroids,

heavy metals, flavonoids, phytoestrogens, dioxin, and various drugs [42]. The increase of AFP levels to 500 ng/ml is correlated with the tumor size; 80% of small HCC show no increase of AFP

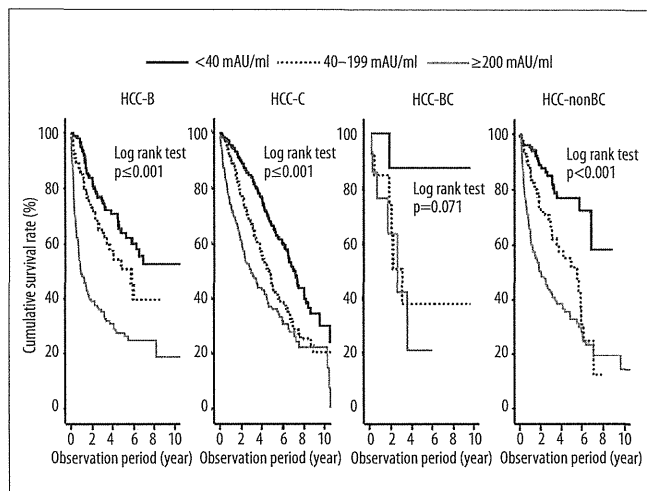


Figure 3. Cumulative survival rate of HCC patients according to DCP levels stratified by chronic viral hepatitis infection.

concentration. Furthermore, sensitivity of AFP decreases from 52% to 25% when tumor diameter is >3 and <3 cm, respectively [42].

There are various differences between DCP and total AFP. Firstly, DCP is a more specific HCC marker than AFP because other liver diseases do not cause an increase of DCP serum levels. DCP measurement for HCC has a sensitivity of 48–62% and a specificity of 81–98% [43]. However, we often encounter patients with liver disease who have slightly elevated DCP levels, but undetectable HCC as assessed by imaging studies. It has been reported that aberrant elevation of DCP is occasionally observed in patients with alcoholic cirrhosis, obstructive jaundice, or vitamin K deficiency [44]. Recently, Toyoda et al. measured a novel DCP (NX-DCP) in serum using a newly developed sandwich ECLIA with new anti-DCP monoclonal antibodies p11 and p16, and reported preliminary data from only 20 HCC patients. They showed that the DCP/NX-DCP ratio may be useful for the diagnosis of HCC among warfarin users [45].

Neither DCP alone nor AFP alone was optimal for the detection of HCC, but the combination of both markers enhanced sensitivity, indicating that these 2 markers are complementary. Several

other studies have shown DCP and AFP to be complementary, which is consistent with the production of DCP and AFP in HCC occurring through different pathways, possibly explaining why sex, race, underlying liver disease, and hepatic disease etiologies had opposite effects on these 2 markers [46–48].

Conclusions

In conclusion, this retrospective cohort study demonstrated DCP to be more sensitive than AFP for the diagnosis of early-stage cryptogenic HCC. We advocate that DCP be used as the main serum test for detecting cryptogenic HCC.

Conflict of interest

The following people have nothing to disclose: Naota Taura, Tatsuki Ichikawa, Hisamitsu Miyaaki, Eisuke Ozawa, Takuya Tsutsumi, Shotaro Tsuruta, Yuji Kato, Takashi Goto, Noboru Kinoshita, Masanori Fukushima, Hiroyuki Kato, Kazuyuki Ohata, Kazuo Ohba, Junichi Masuda, Keisuke Hamasaki, Hiroshi Yatsuhashi, and Kazuhiko Nakao.

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HEPATOLOGY

Significance of hepatitis B virus core-related antigen and covalently closed circular DNA levels as markers of hepatitis B virus re-infection after liver transplantation

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Key words

cccDNA, HBcrAg, HBV, liver transplantation.

Accepted for publication 1 February 2013.

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Conflicts of interest: The authors who have taken part in this study declare that they do not have anything to disclose regarding funding or conflicts of interest with respect to this manuscript.

Abstract

Background and Aim: Currently, hepatitis B virus (HBV) re-infection after liver transplantation (LT) can be almost completely suppressed by the administration of HBV reverse transcriptase inhibitors and hepatitis B immunoglobulins. However, after transplantation, there is no indicator of HBV replication because tests for the serum hepatitis B surface antigen and HBV-DNA are both negative. Therefore, the criteria for reducing and discontinuing these precautions are unclear. In this study, we examined the serum HBV core-related antigen (HBcrAg) and intrahepatic covalently closed circular DNA (cccDNA) in order to determine if these could be useful markers for HBV re-infection.

Methods: Thirty-one patients underwent LT for HBV-related liver disease at Nagasaki University Hospital from 2001 to 2010. Of these, 20 cases were followed up for more than 1 year (median follow-up period, 903 days). We measured serum HBcrAg and intrahepatic cccDNA levels in liver tissue. In addition, in nine cases, we assessed the serial changes of HBcrAg and intrahepatic cccDNA levels from preoperative periods to stable periods.

Results: We examined serum HBcrAg and intrahepatic cccDNA levels in 20 patients (35 samples). HBcrAg and cccDNA levels were significantly correlated with each other ($r = 0.616$, $P < 0.001$). From a clinical aspect, the fibrosis stage was significantly lower in both HBcrAg- and cccDNA-negative patients than in HBcrAg- or cccDNA-positive patients.

Conclusions: HBcrAg and cccDNA were useful as HBV re-infection markers after LT. Keeping patients' HBcrAg and cccDNA negative after LT might contribute to long-term graft survival.

Authors' Contributions:

Toshihisa Matsuzaki: acquisition of data, study concept and design, statistical analysis, writing of manuscript.
Tatsuki Ichikawa: study concept and design, acquisition of data, critical revision of the manuscript for important intellectual content.
Masashi Otani: critical revision of the manuscript for important intellectual content.
Motohisa Akiyama: critical revision of the manuscript for important intellectual content.
Eisuke Ozawa: critical revision of the manuscript for important intellectual content.
Satoshi Miura: critical revision of the manuscript for important intellectual content.
Sadayuki Okudaira: acquisition of data, critical revision of the manuscript for important intellectual content.
Tomayoshi Hayashi: acquisition of data, critical revision of the manuscript for important intellectual content.
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Susumu Eguchi: critical revision of the manuscript for important intellectual content.
Takashi Kanematsu: critical revision of the manuscript for important intellectual content.
Hajime Isomoto: critical revision of the manuscript for important intellectual content.
Fuminao Takeshima: critical revision of the manuscript for important intellectual content.
Kazuhiko Nakao: study supervision, critical revision of the manuscript for important intellectual content.

Introduction

Liver transplantation (LT) is an established procedure for the treatment of end-stage liver disease. However, the recurrence of hepatitis B virus (HBV) is implicated in life-threatening graft failure.¹ Therefore, the prevention of HBV recurrence following LT is a serious concern. The advent of hepatitis B immunoglobulins (HBIG) and the HBV reverse transcriptase inhibitor (RTI) was a major breakthrough in the management of HBV recurrence. Currently, an ideal recurrence rate for HBV has been observed in patients who received HBIG and RTI combination therapy.² However, several studies have reported that HBV can be detected in the transplanted liver and peripheral blood mononuclear cells of recipients even when they have a hepatitis B surface antigen (HBsAg)-negative status.³ Therefore, prophylaxis currently must be continued for the patient's lifetime. However, there are concerns with the long-term administration of HBIG and RTI with respect to safety, medical costs, and resistant mutations of HBV.⁴ In order to discontinue the prophylaxis, several groups have attempted to vaccinate LT recipients against HBV, but most of these studies involve relatively low seroconversion rates because of the immunosuppressive environment.⁵

Recently, new agents against HBV, such as adefovir and entecavir, which hardly develop resistant mutations, have become available. Some have reported that HBIG can be discontinued after LT by using the new anti-HBV agents even if the vaccination does not succeed.⁶ Angus *et al.* reported that when adefovir dipivoxil was substituted for low-dose HBIG, all patients were alive at the study completion without recurrence.⁷ In addition, low-risk cases, such as those with fulminant hepatitis, and hepatitis B core antibody (HBcAb)-positive donors are not necessary for the adminis-

tration of high-dose HBIG.⁸ However, after transplantation, RTI and HBIG may mask the appearance of HBV-DNA, regardless of the presence of intrahepatic HBV covalently closed circular DNA (cccDNA). These factors make it difficult to detect HBV dynamics following LT, and we are therefore unable to determine the feasibility of the discontinuation of prophylaxis.

Recently, a new enzyme immunoassay that detects hepatitis B core-related antigen (HBcrAg) has been reported.⁹ HBcrAg changes in parallel with HBV-DNA in the serum and has a wide detection range.¹⁰ Moreover, its levels are correlated with the intrahepatic cccDNA levels of patients with chronic hepatitis B.¹¹ In addition, we previously reported on the usefulness of HBcrAg in patients receiving anti-HBV prophylaxis following LT.¹²

Therefore, in this study, we simultaneously measured serum HBcrAg and intrahepatic cccDNA levels in liver tissue and studied the HBV dynamics in patients following HBV-related LTs.

Methods

Patients and samples. From 2001 to 2010, a total of 31 patients with HBV-related end-stage liver disease underwent LTs at Nagasaki University Hospital, Nagasaki, Japan. Of these, we enrolled 20 patients who could be followed up for more than approximately 1 year (median 902 days; range 323–2456 days). There were 17 men and 3 women, with a median age of 56.5 years (range 33–68 years). All 20 patients were diagnosed with liver cirrhosis, and 12 were diagnosed with hepatocellular carcinoma. In addition, two patients were coinfectd with the hepatitis C virus (Table 1).

Table 1 Baseline clinical features of the enrolled patients

Case	Age	Gender	Indication disease	HBV-DNA	HBsAg	HBsAb	HBeAg	HBeAb	HBcAb	Donor HBcAb	HBcrAg
1	55	F	LC-B	< 2.6	> 2000	0.2	36.0	0.0	> 100.0	5.0	6.0
2	56	M	LC-B	< 2.6	> 2000	2.3	0.6	82.4	99.9	5.0	4.2
3	48	M	LC-B, HCC	< 2.6	562.5	0.1	1.1	57.7	> 100.0	31.3	5.0
4	60	M	LC-B	< 2.6	1789	0.1	0.2	97.6	> 100.0	70.1	5.8
5	59	M	LC-B, HCC	< 2.6	> 2000	0.1	0.1	> 100.0	> 100.0	5.0	3.2
6	57	M	LC-B, HCC	3.9	188.5	0.5	0.8	54.0	> 100.0	10.3	5.1
7	56	M	LC-B, HCC	< 2.6	> 2000	0.1	1.4	75.4	> 100.0	91.9	5.6
8	68	M	LC-B, HCC	< 2.6	> 2000	0.2	0.1	> 100.0	> 100.0	5.0	3.0
9	33	F	LC-B	3.0	> 2000	0.2	0.2	81.5	99.9	99.6	5.5
10	58	M	LC-B, HCC	3.0	> 2000	0.1	0.1	93.6	> 100.0	93.4	5.1
11	59	M	LC-B	< 2.6	378.3	0.3	0.1	61.6	> 100.0	93.0	3.8
12	57	M	LC-B + C, HCC	< 2.6	519.9	0.1	0.1	> 100.0	99.9	5.0	2.0
13	49	M	LC-B	< 2.6	> 2000	0.1	0.9	52.9	> 100.0	34.1	5.2
14	65	F	LC-B	6.9	> 2000	0.2	0.1	> 100.0	> 100.0	5.0	6.8
15	55	M	LC-B, HCC	< 2.1	> 2000	0.2	0.1	99.3	> 100.0	31.6	4.5
16	46	M	LC-B + C	4.3	1100.4	0.2	0.1	> 100.0	> 100.0	81.9	3.7
17	59	M	LC-B, HCC	< 2.1	> 2000	0.1	0.1	99.2	> 100.0	38.6	3.7
18	51	M	LC-B, HCC	2.1	> 2000	0.2	0.4	62.8	99.4	50.0	4.7
19	67	M	LC-B, HCC	3.9	> 2000	0.1	34.3	60.2	> 100.0	91.1	6.3
20	54	M	LC-B, HCC	2.1	> 2000	0.1	104.8	37.4	> 100.0	9.7	4.3

HBV, hepatitis B virus; HBcAb, hepatitis B core antibody; HBcrAg, hepatitis B core-related antigen; HBeAb, hepatitis B envelope antibody; HBeAg, hepatitis B envelope antigen; HBsAb, antibody against hepatitis B surface antigen; HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LC, liver cirrhosis; LC-B, LC due to HBV; LC-B + C, LC due to HBV-HCV coinfection.

All patients had been receiving RTI since preoperative periods. The HBsAg was negative in all donors, but eight donors were HBcAb-positive (cut-off, 50%), which was suggested to be due to prior exposures to HBV.

The prophylactic infusion of HBIg was administered to all patients according to a fixed-dose schedule; 10 000 units were given intravenously at the anhepatic period during the operation and the next day after the living donor LT (LDLT). Afterwards, 2000 units of HBIg were given routinely in order to keep the serum hepatitis B surface antibody (HBsAb) titers above 100 units/L. After the LDLT, serum HBsAg, hepatitis B envelope antigen (HBeAg), and HBV-DNA were not detected in any of the patients in this study.

Serum samples and biopsy specimens were obtained from 20 patients who received protocol biopsies 1 year after the LDLT at Nagasaki University Hospital after providing informed consent. Nine patients were followed up from the preoperative period to the stable period. Serum samples were obtained at the following three specified intervals: (i) in the preoperative period, samples were obtained just before the operation; (ii) in the postoperative period, samples were obtained during the operation of LT; and (iii) in the stable period, samples were obtained during admission for protocol biopsy. Liver tissue samples were obtained during the following three specified procedures: (i) biopsy from explanted liver during the operation; (ii) time-zero biopsy from the implanted liver during the operation; and (iii) protocol biopsy 1 year after the LDLT.

Serological markers for HBV. HBsAg, HBsAb, HBeAg, hepatitis B envelope antibodies (HBeAb), and HBcAb levels were assessed by the chemiluminescence enzyme immunoassay (CLEIA) method using a commercially available enzyme immunoassay kit (Lumipulse, Fuji Rebio, Inc., Tokyo, Japan). Serum concentrations of HBV-DNA were determined using a polymerase chain reaction (PCR) HBV monitoring kit (Roche Diagnostics K.K., Tokyo, Japan), which had a quantitative range from 2.6 to 7.6 log copies/mL.

HBcrAg test. Serum HBcrAg levels were measured by a CLEIA HBcrAg assay kit (Fujirebio, Inc.) with a fully automated analyzer system (Lumipulse System, FujiRebio, Inc.). HBcrAg concentrations were expressed as units/mL (U/mL). In this study, HBcrAg values were expressed as log U/mL, and the cut-off value was set at 3.0 log U/mL.^{9,13}

Measurement of cccDNA. Liver tissues were stored at -80°C before DNA extraction. HBV-DNA was extracted using a high pure PCR template preparation kit (Roche Diagnostics K.K.). The concentration of purified DNA was measured at an absorbance of 260 nm.

cccDNA levels were measured with the real-time PCR method. With reference to a previous study,¹¹ we designed two oligonucleotide primers, cccF2 (5'-CGTCTGTGCCTTCTCATCTGA-3', nucleotides: 1424-1444) and cccR4 (5'-GCACAGCTTGGAGGC TTGAA-3', nucleotides: 1755-1737), and a cccP2 probe (5'-FAM-ACCAATTTATGCCTACAG-MGB-3', nucleotides: 1672-1655). Reaction volume (20.0 μL) containing 500 ng of extracted DNA,

0.5 $\mu\text{mol/L}$ of each primer, 0.2 $\mu\text{mol/L}$ of the probes, and Light-Cycler TaqMan Master (Roche Diagnostics K.K.) was administered. The initial activation step was heated at 95°C for 10 min. The subsequent PCR conditions consisted of 60 cycles of denaturation at 95°C for 10 s, and annealing and extension at 60°C for 30 s per cycle. Real-time PCR was performed in a LightCycler (Roche Diagnostics K.K.). Serial dilutions of a plasmid containing an HBV monomer were used as quantitation standards.

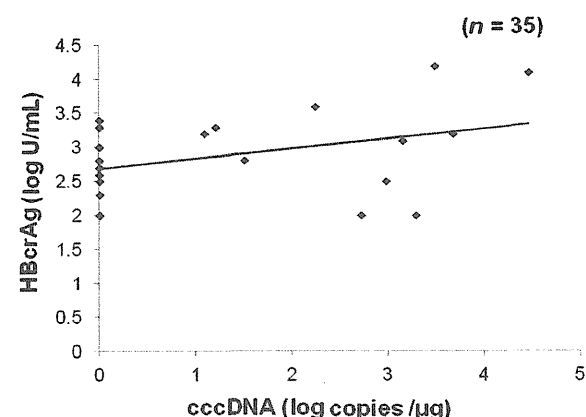
Liver histology. Liver histology was evaluated by the same two pathologists. The degrees of necroinflammation and fibrosis were assessed based on the New Inuyama classification.¹⁴ The degrees of rejection were assessed with the Rejection Activity Index according to the Banff working classification of hepatic allograft pathology.¹⁵

Liver function test. Blood biochemical tests were performed in all patients, and liver function was evaluated. Liver function was assessed using Pugh's modification of Child's scoring system.¹⁶

Statistical analyses. Student's *t*-tests and Fisher's exact tests were used for comparisons between groups of parametric quantitative data, and Mann-Whitney *U*-tests were used for comparisons between independent groups of non-parametric data. Categorical variables were compared with chi-square tests. The correlations between continuous variables were analyzed by the Pearson's correlation test. Two-tailed *P* values less than 0.05 were considered statistically significant.

Results

Correlation between HBcrAg and cccDNA. The correlation between HBcrAg and cccDNA levels in all 35 samples is summarized in Figure 1. A statistically significant positive



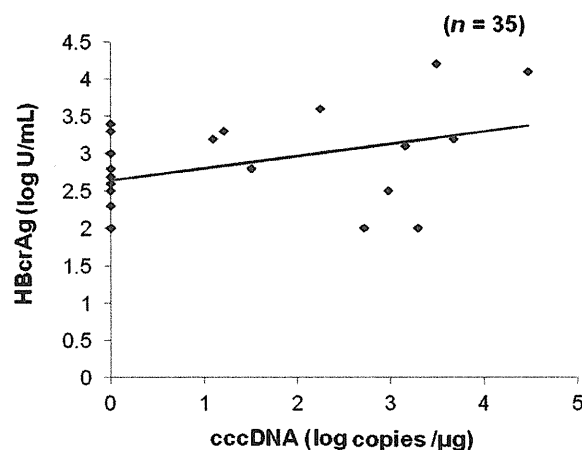


Figure 2 Correlation between hepatitis B core-related antigen (HBcrAg) and covalently closed circular DNA (cccDNA) levels after transplantation. $r = 0.402$, $P = 0.046$ ($y = 0.16x + 2.64$). Straight lines indicate the correlation between HBcrAg and cccDNA levels.

correlation was observed ($r = 0.616$, $P < 0.001$). Similarly, in the 23 samples that were obtained after LT only (that is, preoperative state samples were excluded), HBcrAg levels were significantly correlated with cccDNA levels (Fig. 2, $r = 0.402$, $P = 0.046$). These results supported the hypothesis that HBcrAg can be useful as an HBV marker instead of cccDNA after LT.

Serial changes in HBcrAg and cccDNA levels. HBcrAg and cccDNA levels showed similar dynamics during each period (Figs 3,4). All nine cases had positive levels of HBcrAg. However, seven of them were negative for HBV-DNA. During the post-transplantation period, HBcrAg levels of seven cases and cccDNA levels of eight cases became negative. Subsequently, HBcrAg and cccDNA levels of five cases became positive again during the stable period. These dynamics implicated the re-infection of HBV in the graft liver.

Comparisons of the clinical features of HBcrAg and cccDNA levels. We divided patients into two groups according to their status of HBcrAg and cccDNA, and investigated their clinical features (Table 2). Positive group includes the patients with positive cccDNA or HBcrAg, negative group includes the patients with both negative.

In comparisons between the positive group and negative group, the number of patients being treated with entecavir was significantly lower in negative group ($P = 0.022$). Additionally, the stage of the graft liver was significantly lower ($P = 0.012$) in negative group. The grafts of the HBcrAg- and cccDNA-negative patients were in good condition in the lower fibrosis stages (median 0; range 0–1).

Discussion

In the present study, we demonstrated the usefulness of HBcrAg and cccDNA as markers of HBV after transplantation. As in our

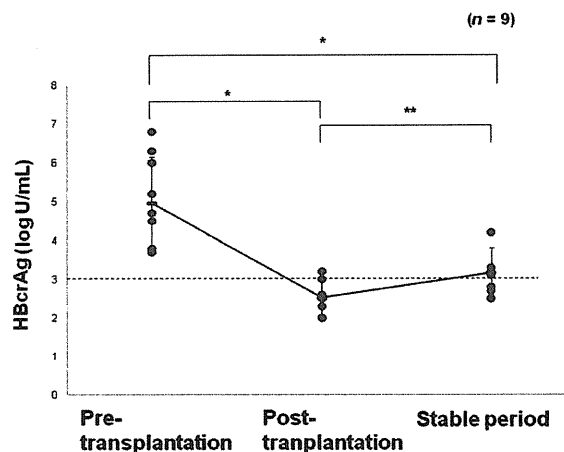


Figure 3 Serial changes of the hepatitis B core-related antigen (HBcrAg) levels. HBcrAg levels are represented as mean values; the closed circles show the values of the HBcrAg levels in all phases. The error bars indicate standard deviations. The detection range is above 3.0 log U/mL. In order to obtain the mean value, the values of 3.0 log U/mL or less, and 2.0 log U/mL or more were added to the calculation. The mean values of HBcrAg levels dropped during the postoperative period but then gradually increased again during the stable period (* $P < 0.001$ and ** $P = 0.035$ indicate the significant differences between each period).

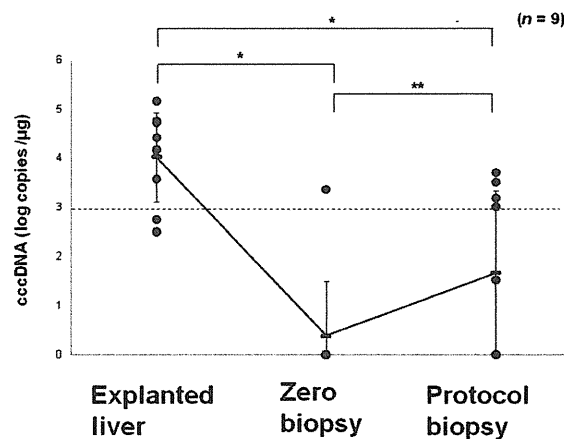


Figure 4 Serial changes of the covalently closed circular DNA (cccDNA) levels. cccDNA levels are represented as mean values; the closed circles show the values of the cccDNA levels in all phases. The error bars indicate standard deviations. The mean values of the cccDNA levels dropped during the time-zero biopsy but then gradually increased during the protocol biopsy (* $P < 0.001$ and ** $P = 0.078$ indicate the significant differences between each period).

previous report,¹² we suggest that HBcrAg, which is a newly developed enzyme immunoassay,⁹ is a possible method for detecting the dynamics of HBV after LT. However, HBcrAg consists of HBcAg, HBeAg, and p22cr, which is generated from cccDNA,

Table 2 Comparisons of the clinical features of HBcrAg and cccDNA levels

HBcrAg/cccDNA status	Positive group	Negative group	Positive versus negative
Patient M/F	10/2	7/1	NS
Day after transplantation [†]	854 (323–2163)	1674.5 (353–2456)	NS
Age [†]	55.5 (33–68)	56.5 (48–65)	NS
Serum HBV-DNA positive at LT (p/n)	7/5 (58.3%)	2/6 (33.3%)	NS
Serum HBeAg positive at LT (p/n)	1/11 (8.3%)	1/7 (14.3%)	NS
HBcAb-positive donor (p/n)	7/5 (58.3%)	1/7 (14.3%)	NS
Blood incompatibly (p/n)	1/11 (8.3%)	1/7 (14.3%)	NS
Presence of HCC at LT (p/n)	9/3 (75%)	7/1 (87.5%)	NS
RTI for prophylactic therapy after LT			
Use of LAM	3/12 (25%)	4/8 (50%)	NS
Use of ETV	9/12 (75%)	1/8 (12.5%)	<i>P</i> = 0.022
Use of ADV	0 (0%)	2/8 (25%)	NS
Use of LAM + ADV	0 (0%)	1/8 (12.5%)	NS
Immunosuppression after LT			
Use of TAC	10/12 (83.3%)	5/8 (62.5%)	NS
Use of CYA	0 (0%)	2/8 (25%)	NS
Use of MMF	2/12 (16.6%)	0 (0%)	NS
Use of TAC + MMF	0 (0%)	1/8 (12.5%)	NS
Liver function test			
Serum albumin (g/L) [‡]	39.2 (4.7)	40.0 (4.8)	NS
Child–Pugh score [‡]	5.0 (5.0–9.0)	5.0 (5.0–6.0)	NS
Histology of LB			
Grade [‡]	1.0 (0.0–3.0)	0.5 (0.0–1.0)	NS
Stage [‡]	1.0 (0.0–3.0)	0.0 (0.0–1.0)	<i>P</i> = 0.0027
RAI score [‡]	2.5 (0.0–5.0)	1.5 (0–4)	NS

Fisher's exact test for categorical variables.

[†]Mann–Whitney *U*-test for non-normally distributed variables, expressed as median (range).[‡]Student's *t*-test for normally distributed variables, expressed as mean (SD).

ADV, adefovir; cccDNA, covalently closed circular DNA; CYA, cyclosporin A; ETV, entecavir; HBV, hepatitis B virus; HBcAb, hepatitis B core antibody; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B envelope antigen; HCC, hepatocellular carcinoma; LAM, lamivudine; LB, liver biopsy; LT, liver transplantation; MMF, mycophenolate mofetil; n, negative; NS, not significant; p, positive; RAI, Rejection Activity Index; RTI, reverse transcriptase inhibitor; SD, standard deviation; TAC, tacrolimus.

and thus, it is questionable if HBcrAg truly reflects the viral pattern of HBV. Therefore, we designed this study to examine the usefulness of further analysis of cccDNA, which truly functions as a reservoir of HBV replication.

In the results of this study, a positive correlation between HBcrAg and cccDNA was shown, and this was consistent with a previous report on chronic hepatitis B.¹¹ These findings suggest the usefulness of monitoring HBV dynamics of patients after LTs because examinations of serum HBcrAg are less invasive methods compared with examinations of cccDNA levels in liver tissue. HBcrAg enables us to frequently check the HBV dynamics of patients, and it contributes to a reduction in the risk of HBV reactivation.¹³

However, as shown in Table 2, the results of the HBcrAg and cccDNA levels were not matched in 35% (7 of 20) of the patients. This may be due to a problem with the sensitivity of these two markers. We should use these markers cautiously because HBV might exist even if these were negative. Suzuki *et al.* reported that among the 13 patients with negative results for HBsAg, HBeAg, and HBV-DNA, all had positive results with cccDNA, while HBcrAg was positive in only seven patients.¹¹ In addition, cccDNA was also examined in a limited way because it was

extracted from tissue from only a small part of the liver. Moreover, some reports have suggested that cccDNA can be detected in extrahepatic sites,¹⁷ and thus, it is impossible to determine whether HBV exists with only one method. Therefore, we preferred to assess HBV dynamics with these two methods in order to overcome problems with sensitivity.

Interestingly, in the group with negative results for both of the two markers, the fibrosis stage was significantly lower compared with the other. This might reflect HBV activity after the LT. In addition, it was considered that keeping the two markers negative after LT may suggest the possibility of an extension of graft survival. But we observed only a limited period, further study of long-term outcome will be required.

The goal of this study was to determine the criteria for the appropriate prophylaxis of HBV related to LT with these two markers. Lenci *et al.* reported that 80.1% of the patients with undetectable intrahepatic cccDNA levels did not exhibit signs of HBV recurrence, even after withdrawal of the prophylaxis.¹⁸ We thought that it might be possible to select patients more efficiently and correctly by using a method that combines examinations of HBcrAg and cccDNA. We observed one patient with both HBcrAg- and cccDNA-negative discontinued antiviral therapy.

Although the patient stopped antiviral therapy, he has not relapsed for 29 months (data not shown).

In conclusion, HBcrAg and cccDNA were helpful for the monitoring of HBV dynamics after LT and keeping a negative status of these markers might contribute to graft survival. In addition, using these methods, the criteria for the discontinuation of HBV prophylaxis could be clarified in the future.

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Review Article

Recent topics on α -fetoprotein

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Zinc-fingers and homeoboxes 2 (ZHX2) and zinc-finger and BTB domain containing 20 (ZBTB20) repress the postnatal expression of α -fetoprotein (AFP) by interacting with the AFP gene promoter regions. ZHX2 inhibits the expression of AFP and cyclins A and E. ZBTB20 is negatively regulated by CUX1, which promotes cell-cycle progression, suggesting that AFP reactivation is closely linked to hepatocyte proliferation. A slight elevation in the serum AFP level often occurs in patients with chronic hepatitis C in the absence of hepatocellular carcinoma (HCC) and is an independent risk factor for HCC development to complement the fibrosis stage. In addition, the sustained elevation of AFP after interferon therapy is a risk factor of HCC development. AFP levels are clinically useful in predicting the outcomes of liver transplantation and sorafenib therapy for HCC patients. A low preoperative AFP level

is a predictor of long-term survival and is associated with a low recurrence rate of HCC after liver transplantation. AFP response ($\geq 20\%$ decrease in AFP during 6–8 weeks of treatment) rather than radiological outcomes is a significant prognostic factor for survival in sorafenib-treated HCC patients. Highly sensitive *Lens culinaris* agglutinin-reactive AFP (AFP-L3) is 5–10 times more sensitive than conventional AFP-L3, and useful for early detection of HCC in patients with total AFP below 20 ng/mL.

Key words: α -fetoprotein, chronic hepatitis C, hepatocellular carcinoma, highly sensitive *Lens culinaris* agglutinin-reactive α -fetoprotein, liver transplantation, sorafenib

INTRODUCTION

THE α -FETOPROTEIN (AFP) and albumin genes are similar in structure and tandemly arranged on the q arm of chromosome 4. Both genes are expressed at high levels in fetal liver. After birth, AFP expression decreases rapidly to an almost undetectable level, whereas albumin expression remains high.^{1,2} The AFP gene is reactivated in pathological conditions such as hepatocellular carcinoma (HCC). The release of AFP gene repression in hepatocytes may be linked to hepatocarcinogenesis. The serum level of AFP is elevated in benign liver diseases, such as chronic viral hepatitis and liver cirrhosis without HCC.^{3,4} Elevated AFP levels are linked to alanine aminotransferase elevation, hepatocyte regeneration and hepatic fibrosis.^{3,4} A rising level of AFP over the first few hospital days indicates a better prog-

nosis of acute liver failure.⁴ In the present study, we reviewed the relationship between clinical features and serum AFP levels in patients with chronic hepatitis C (CHC) without HCC.

Assessment of AFP response after locoregional therapy for HCC, including surgical resection, radiofrequency ablation and transcatheter arterial chemoembolization, is simple and sensitive for detecting radiological tumor response, as well as an early objective screening tool for progression by imaging.^{5,6} We reviewed the clinical usefulness of monitoring the serum AFP level during two newly established therapies for HCC, liver transplantation and administration of sorafenib. Finally, clinical usefulness of highly sensitive *Lens culinaris* agglutinin-reactive AFP (hs-AFP-L3) for detection and management of HCC is discussed.

AFP GENE REGULATION

THE AFP GENE is positively regulated by transcription factors including HNF-1, HNF-3, HNF-4 and C/EBP that bind to specific elements in the promoter and enhancer regions.^{7–9} These factors also bind to regulatory regions of the albumin gene,^{10,11} which is consti-

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Received 13 September 2012; revision 6 December 2012; accepted 20 December 2012.

tutively expressed in adult liver. Therefore, the existence of factors that specifically silence the AFP gene in adult liver has been supposed.^{1,2,7–11} Indeed, two novel factors involved in postnatal AFP silencing have been identified, zinc-fingers and homeoboxes 2 (ZHX2)¹² and zinc-finger and BTB domain containing 20 (ZBTB20).¹³

In a study of the hereditary persistence of AFP in the liver of BALB/c mice, ZHX2 was identified as a postnatal repressor of AFP expression.¹² Shen *et al.* showed that ZHX2 overexpression decreases AFP secretion in human hepatoma cells expressing high AFP levels and that ZHX2 repression is governed by the human AFP promoter and requires intact HNF1 binding sites.¹⁴ Hypermethylation of CpG islands in the ZHX2 promoter and the resultant loss of ZHX2 expression were detected in human HCC tissues, but not in surrounding non-tumor tissues.¹⁵ These data suggest that ZHX2 contributes to human AFP repression in adult liver and may be involved in AFP reactivation in HCC. ZHX2 also represses glypican 3, an oncofetal gene.¹⁶ Yue *et al.* reported that ZHX2 inhibits HCC cell proliferation by preventing the expression of cyclins A and E (Fig. 1a) and reduces the growth of xenograft tumors in mice.¹⁷ Thus, they proposed that the loss of nuclear ZHX2 may be an early step in the development of HCC.

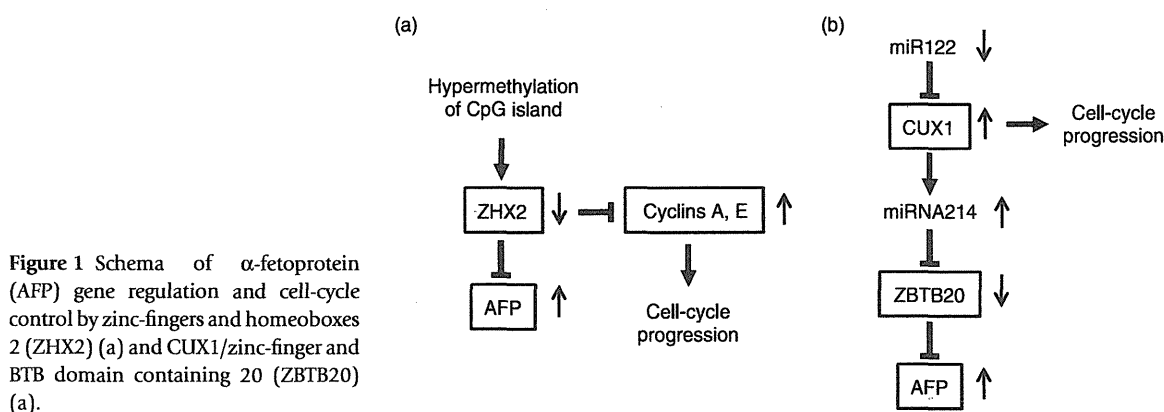
ZBTB20 is another repressor of AFP gene transcription in the liver. The main isoforms of ZBTB20 in humans and mice are 741 and 733 amino acids in length.⁹ The liver-specific deletion of ZBTB20 resulted in the persistence of AFP expression in adult mouse liver.¹³ ZBTB20 directly binds to a region of the AFP promoter between –108 and –53 and represses the AFP promoter activity.¹³ Recently, it was shown that miR122, a liver-specific miRNA, indirectly modulates the expression of ZBTB20 and regulates AFP expression.¹⁸ The miR122-silenced

HCC cells exhibit a more invasive phenotype and produce more abundant AFP. In the miR122-silenced cells, the expression of CUX1, a transcription factor that regulates multiple processes including cell-cycle progression, is upregulated. CUX1 is a positive regulator of miR214. Because ZBTB20 is a target of miR214, the elevated expression of miR214 represses the ZBTB20 translation, followed by increased expression of AFP (Fig. 1b).¹⁸

Accordingly, ZHX2 inhibits the expression of both AFP and cyclins A and E.¹⁷ ZBTB20 inhibits AFP expression and is regulated by CUX1, which promotes cell-cycle progression.¹⁸ These findings suggest that AFP reactivation is closely linked to hepatocyte proliferation (Fig. 1).

AFP ELEVATION IN CHRONIC HEPATITIS C

MILDLY ELEVATED SERUM AFP levels are often seen in patients with CHC without HCC. The clinical significance of this mild elevation in serum AFP has been investigated. Hu *et al.* reported that in 357 patients with CHC without HCC, 82 (23.0%) patients had AFP levels of 10 ng/mL or more, and the AFP elevation was independently associated with stage III/IV hepatic fibrosis, the serum level of aspartate aminotransferase (AST) and prolonged prothrombin time.³ The prevalence of elevated AFP (≥ 10 ng/mL) was 15.3% (28/183), 24.5% (25/102) and 42.0% (29/69) in stages 0–II, III and IV hepatic fibrosis, respectively.³ In another report, elevated AFP levels (≥ 15 ng/mL) were observed in 23.9% (156/654) of CHC patients, and thrombocytopenia, AST elevation and AFP levels of 6 ng/mL or more were associated with advanced hepatic fibrosis.⁴ Richardson *et al.* analyzed 258 275 AFP tests in a cohort



of 76 347 hepatitis C virus (HCV)-infected patients.¹⁹ Of these, 12 775 (16.6%) patients had cirrhosis, and 1488 (1.9%) patients developed HCC during the observation period. Among patients without HCC, significant determinants for increased levels of AFP included cirrhosis, high Model for End-Stage Liver Disease (MELD) score, and increased level of alanine aminotransferase.¹⁹ Tateyama *et al.* reported that a slightly elevated AFP level is an independent risk factor for HCC to complement the fibrosis stage in a retrospective study of 707 CHC patients without HCC.²⁰ The 10-year cumulative incidence rates of HCC in patients with AFP levels of less than 6, 6–20 and 20 mg/mL or more at entry were 6.0%, 24.6% and 47.3%, respectively.²⁰

In addition, the change in AFP level during interferon (IFN) therapy in CHC patients has been investigated. The serum level of AFP before pegylated (PEG) IFN/ribavirin (RBV) therapy predicts treatment outcome in CHC patients regardless of HCV genotype.²¹ The serial AFP levels decreased after PEG IFN/RBV treatment, presenting in a time-dependent manner, specifically in patients who achieved a sustained virological response.²² A decrease of serum AFP level after low-dose IFN therapy regardless of virological response has also been reported.^{23–25} Moreover, Tamura *et al.* reported that increased serum AFP levels (≥ 10 ng/mL) at the end of IFN therapy was a significant variable affecting the development of HCC.²⁶ Osaki *et al.* also reported that among patients without a sustained virological response, a decrease in the AFP value (< 10 mg/mL) by IFN therapy correlates with a reduced risk of HCC incidence after treatment.²⁷ Taken together, the results of these studies indicate that the sustained elevation of AFP (≥ 10 ng/mL) after IFN therapy is a risk factor of HCC development. In this regard, Akuta *et al.* reported that the substitution of amino acid 70 in the HCV core region of genotype 1b is an important predictor of elevated AFP in CHC patients without HCC.²⁸ The substitution of amino acid 70 in the HCV core region is related to non-sustained virological response by IFN therapy²⁹ and also to hepatocarcinogenesis.^{30,31}

AFP LEVELS IN LIVER TRANSPLANTATION FOR HCC

THE SERUM LEVEL of AFP before liver transplantation for HCC is clinically significant. Several studies found that a low preoperative AFP level is a predictor of long-term survival and associated with a low recurrence rate of HCC after liver transplantation.^{32–37} Mailey *et al.* analyzed 2253 patients who underwent orthotopic

liver transplantation.³⁸ In this patient group, 1210 (53.7%), 805 (35.7%) and 238 (10.6%) patients had low (< 20 ng/mL), medium (20–399 ng/mL) or high (≥ 400 ng/mL) AFP levels, respectively. The low AFP group had the greatest 4-year survival rate (76%) as compared to the medium (65%; $P < 0.001$) and high (57%; $P < 0.001$) AFP groups, and the improved survival in the low AFP group was still observed in patients with only stage II HCC.³⁸ Todo *et al.* analyzed a total of 653 patients with HCC who received living donor liver transplants in Japan.³⁹ In this study, the preoperative serum AFP levels were inversely correlated with patient survival: 83.8% at 1 year, 77.3% at 3 years and 72.2% at 5 years when AFP was less than 200 ng/mL ($n = 473$), and 64.9% at 1 year, 42.5% at 3 years and 34.0% at 5 years when AFP was 1000 ng/mL or more ($n = 48$).³⁹ Wang *et al.* reported 1-, 2- and 3-year recurrence-free survival rates of 83%, 63% and 53%, respectively, for patients with AFP levels of less than 20 ng/mL.⁴⁰ These survival rates were much greater than the corresponding rates for patients with AFP levels of 700 ng/mL or more (68%, 49% and 32% for the 1-, 2- and 3-year recurrence-free survival rates, respectively).⁴⁰ Fujiki *et al.* studied 144 HCC patients who received living donor liver transplants.⁴¹ The 1-, 3- and 5-year recurrence-free survival rates for patients with AFP levels were less than 200 ng/mL in comparison to patients with AFP levels of 800 ng/mL or more were 97% versus 65%, 91% versus 40% and 90% versus 40%, respectively. However, the preoperative level of des- γ -carboxy prothrombin (DCP) (≥ 400 mAU/mL) was a stronger predictor of recurrence than the AFP level (≥ 800 ng/mL), and the DCP level (≥ 400 mAU/mL) was significantly related to microvascular invasion and poor differentiation of HCC cells.⁴¹

Pretransplant treatment of HCC patients with high AFP levels could be feasible and lead to similar intent-to-treat and post-transplant survival rates to those of patients with persistently low AFP levels.⁴² Merani *et al.* analyzed 6871 HCC patients listed for liver transplantation and reported that patients with AFP levels decreased to 400 ng/mL or less by local pretransplant HCC treatment and patients with AFP levels persistently 400 ng/mL or less had similar dropout rates from the transplant list (10% for both groups) and similar post-transplant survival rates (89% vs 78% at 3 years, $P = 0.11$).⁴³ They concluded that only the last pretransplant AFP level independently predicted survival ($P < 0.001$), unlike the AFP level at the time of listing or AFP changes.⁴³ Toso *et al.* analyzed 5498 adult candidates for liver transplantation for HCC and 43 528 liver

transplant candidates with a non-HCC diagnosis.⁴⁴ They found that the dropout risk of HCC patients was predicted by the MELD score, HCC size, HCC number and AFP, and they calculated the dropout equivalent MELD (deMELD) points that express similar risks of dropout between HCC and non-HCC patients and allow for the management of both groups on a common waiting list.⁴⁴ The deMELD equation was obtained as follows:

$$\begin{aligned} \text{deMELD} = & -25 + 0.1 \times \text{age} + 1.6 \times \text{MELD} + 1.6 \\ & \times \text{tumor size} + 1.3 \times \log(\text{AFP}), +6 \text{ if tumor number} \\ & \geq 2, +0 \text{ if diagnosis} = \text{HCV}, -1 \text{ if diagnosis} \\ & = \text{hepatitis B virus}, +3 \text{ if diagnosis} = \text{alcohol}, +3 \text{ if} \\ & \text{diagnosis} = \text{non-alcoholic steatohepatitis}, +1 \text{ if} \\ & \text{diagnosis} = \text{hemochromatosis}, +1 \text{ if diagnosis} = \text{other}. \end{aligned}$$

AFP LEVELS IN SORAFENIB-TREATED HCC PATIENTS

SORAFENIB IS AN antiangiogenic agent used to treat advanced HCC.^{45,46} Sorafenib sometimes induces disappearance of contrast enhancement of HCC at the arterial phase, but rarely induces HCC shrinkage. Therefore, it is difficult to evaluate the antitumor effect of sorafenib or to predict its survival effect by imaging-based Response Evaluation Criteria in Solid Tumors (RECIST). Personeni *et al.* investigated the prognostic usefulness of a decrease in serum AFP levels and compared it to RECIST in 82 HCC patients treated with sorafenib.⁴⁷ AFP response ($\geq 20\%$ decrease in AFP during 8 weeks of treatment) rather than the radiological outcomes evaluated by RECIST was a significant prognostic factor for survival in multivariate analysis. The authors concluded that the assessment of AFP response was superior to RECIST in determining the response to sorafenib treatment.⁴⁷ Similarly, Yau *et al.* reported that decreased AFP levels ($\geq 20\%$ of the baseline level after 6 weeks of sorafenib) were significantly associated with progression-free survival both in 41 exploration patients and 53 validation patients.⁴⁸ When Kuzuya *et al.* evaluated the relationships between antitumor response based on imaging studies and early changes (2 and 4 weeks after starting sorafenib therapy) in AFP and DCP levels, they found that a significant early decrease in AFP levels was observed in the partial response and stable disease groups, while DCP levels increased despite therapeutic efficacy.⁴⁹ The authors speculated that the ischemic change of HCC cells may result in the elevation of DCP level, and concluded that AFP levels rather than DCP levels are useful for predicting antitumor responses during sorafenib therapy. In addition, the retrospective

analysis of 66 patients treated with sorafenib revealed that assessment of overall survival by a change in AFP ratio of 1 or less at 8 weeks was better than that of more than 1 at 8 weeks ($P = 0.002$), but DCP ratio was not useful for assessment of overall survival.⁵⁰

HS-AFP-L3

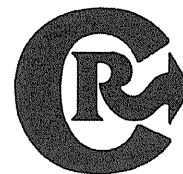
LENS CULINARIS AGGLUTININ-REACTIVE AFP, a fucosylated fraction of AFP, is a highly specific marker for HCC compared with AFP, and its elevation links to poor prognosis.⁵¹ However, the advantage of AFP-L3 measured by conventional method had been limited due to its low sensitivity, especially in patients with total AFP below 20 ng/mL. To resolve this issue, a hs-AFP-L3 assay has been recently developed. There are several studies supporting the clinical utility of a newly developed hs-AFP-L3 assay in patients with low total AFP levels.^{52–56} Toyoda *et al.* reported that sensitivity and specificity of hs-AFP-L3 for HCC in patients with total AFP below 20 ng/mL was 25–50% and more than 85%, respectively, at the cut-off level between 5% and 7%.⁵⁷ These results suggest that hs-AFP-L3 is 5–10-times more sensitive than conventional AFP-L3, maintaining high specificity. They proposed that hs-AFP-L3 elevation in patients with total AFP level below 20 ng/mL indicates poor prognosis of HCC and predicts detection of HCC in high-risk patients under surveillance. Thus it is possible that hs-AFP-L3 is a useful biomarker for detection and management of HCC, especially in patients with low total AFP.

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Injectable hyaluronic acid-tyramine hydrogels incorporating interferon- α 2a for liver cancer therapy

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ARTICLE INFO

Article history:

Received 16 November 2012

Accepted 5 January 2013

Available online 14 January 2013

Keywords:

Hydrogel

Injectable

Protein delivery

Hyaluronic acid

Interferon

ABSTRACT

We report an injectable hydrogel system that incorporates interferon- α 2a (IFN- α 2a) for liver cancer therapy. IFN- α 2a was incorporated in hydrogels composed of hyaluronic acid-tyramine (HA-Tyr) conjugates through the oxidative coupling of Tyr moieties with hydrogen peroxide (H_2O_2) and horseradish peroxidase (HRP). IFN- α 2a-incorporated HA-Tyr hydrogels of varying stiffness were formed by changing the H_2O_2 concentration. The incorporation of IFN- α 2a did not affect the rheological properties of the hydrogels. The activity of IFN- α 2a was furthermore well-maintained in the hydrogels with lower stiffness. Through the caspase-3/7 pathway *in vitro*, IFN- α 2a released from HA-Tyr hydrogels inhibited the proliferation of liver cancer cells and induced apoptosis. In the study of the pharmacokinetics, a higher concentration of IFN- α 2a was shown in the plasma of mice treated with IFN- α 2a-incorporated hydrogels after 4 h post injection, with a much higher amount of IFN- α 2a delivered at the tumor tissue comparing to that of injecting an IFN- α 2a solution. The tumor regression study revealed that IFN- α 2a-incorporated HA-Tyr hydrogels effectively inhibited tumor growth, while the injection of an IFN- α 2a solution did not demonstrate antitumor efficacy. Histological studies confirmed that tumor tissues in mice treated with IFN- α 2a-incorporated HA-Tyr hydrogels showed lower cell density, with more apoptotic and less proliferating cells compared with tissues treated with an IFN- α 2a solution. In addition, the IFN- α 2a-incorporated hydrogel treatment greatly inhibited the angiogenesis of tumor tissues.

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1. Introduction

Therapeutic proteins are a major class of pharmaceuticals. As of 2010, there had been 200 protein based products approved for therapeutic applications with more than 600 under development [1]. Protein therapeutics consist of monoclonal antibodies and vaccines, as well as hormones, growth factors, cytokines, enzymes, etc. They can provide treatments for a variety of diseases, ranging from microbial infection [2] to autoimmune diseases [3] and cancer [4]. One of the greatest advantages of using protein therapeutics is the capacity to produce high affinity and specificity antibodies to virtually any target of interest, through transgenic mice and/or phage display technologies [5]. Yet many of these protein drugs, with the exception of whole antibodies and Fc-fusion proteins [6], possess a rather short terminal half-life in the range of minutes to hours. These proteins usually undergo rapid clearance by peripheral blood-mediated proteolysis, renal and hepatic elimination, as well as elimination by receptor-mediated endocytosis [6]. In order to maintain an effective concentration of protein drugs during therapy, frequent infusions are applied that often lead to various side-effects and discomfort for patients [7].

Thus, a drug delivery system for the prolonged release of protein therapeutics is highly desirable.

The use of hydrogels as depots for prolonged drug release has been extensively studied. As hydrogels imbibe large amounts of water, they can provide an aqueous environment for protein therapeutics and prevent their denaturation [8]. An injectable hydrogel system is particularly useful in drug delivery as surgery is not required for implantation. Recently, an enzymatic crosslinking strategy has attracted intensive attention in the area of drug delivery and regenerative medicine [9,10]. We have previously reported an injectable and biodegradable hydrogel system composed of hyaluronic acid-tyramine conjugates (HA-Tyr) [11,12]. The hydrogels were formed through the oxidative coupling reaction of the Tyr moieties, catalyzed by hydrogen peroxide (H_2O_2) and horseradish peroxidase (HRP).

The advantage of the enzymatically-crosslinked HA-Tyr hydrogel system is the independent tuning of hydrogel stiffness and gelation rate [13]. The stiffness of HA-Tyr hydrogels could be controlled by the concentration of H_2O_2 while the gelation rate was tuned by the concentration of HRP. It was previously demonstrated that model proteins, such as lysozyme and α -amylase, could be incorporated and subsequently released from HA-Tyr hydrogels. Moreover, the protein release profile and the activity of released proteins were

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shown to depend on the stiffness of the hydrogel as well as the isoelectric point of the protein [14]. Other *in situ* forming HA-based hydrogel systems, such as thiolated HA which crosslink via poly(ethylene glycol) diacrylate (PEGDA) by addition reaction [15,16] or furan-modified HA which crosslink via dimaleimide poly(ethylene glycol) by Diels–Alder reaction [17], require extensive modifications to HA with the degree of substitution between 40 to 60%. By comparison, the degree of substitution of HA-Tyr is relatively low (4–6%) and yet hydrogels can be formed rapidly by the enzyme-mediated oxidative reaction. Indeed, it was demonstrated that HA-Tyr hydrogels with a gel point of 1–2 min could effectively incorporate proteins within the gel matrix and thus prevent undesired leakage of the gel precursors and proteins into the surrounding tissue.

From these perspectives, we consider that the design of an injectable hydrogel system that incorporated protein therapeutics would be important for the treatment of diseases. Herein, the anticancer effect of IFN- α 2a-incorporated HA-Tyr hydrogels was explored *in vitro* and *in vivo* and compared to the effect of IFN- α 2a solution. IFN- α 2a, one of the IFN analogues, is a class of cytokines with various functions, the most well-known of which is its anti-viral activity [18,19]. In recent years, there are increasing numbers of reports that demonstrate IFN inhibits the proliferation and induces apoptosis of cancer cells in hepatocellular carcinoma [20,21]. In this study, we first prepared IFN- α 2a-incorporated HA-Tyr hydrogels with varying stiffness and studied the release of the protein from hydrogels *in vitro*. After that, we examined the activity of proteins incorporated in the hydrogels using cells with subgenomic Hepatitis C virus (HCV) replicon. Then, we confirmed the inhibition of proliferation and induction of apoptosis of human liver cancer cells HAK-1B with IFN-incorporated HA-Tyr hydrogels *in vitro*. In an animal experiment, we studied the pharmacokinetics of the protein drug in the plasma of hydrogel-treated mice and also the amount of IFN- α 2a delivered at the tumor tissue. Finally, we evaluated the efficacy of IFN- α 2a-incorporated HA-Tyr hydrogels in tumor regression with a HAK-1B-xenografted nude mice model. The histology and immunohistochemistry of tumor tissues were also characterized on mice treated with PBS, IFN- α 2a alone or IFN- α 2a-incorporated hydrogels.

2. Materials and methods

2.1. Materials and cell culture

Sodium hyaluronate (HA) (MW = 90 kDa, density = 1.05 g/cm³) was kindly donated by JNC Corporation (Tokyo, Japan). Hyaluronidase (439 units/mg) from bovine testes was purchased from Sigma-Aldrich (Singapore). Horseradish peroxidase (HRP, 100 units/mg) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Interferon α 2a (IFN- α 2a, 1×10^8 IU/mg protein) was purchased from Santa Cruz (CA, USA). VeriKine™ Human Interferon-Alpha ELISA kit was purchased from PBL Interferon Source (NJ, USA). Luciferase assay system and Apo-ONE® homogeneous caspase-3/7 assay kit were purchased from Promega (Singapore). AlamarBlue assay kit, Alexa Fluor® 488 annexin V/Dead cell apoptosis kit for flow cytometry and Image-iT™ live red caspase detection kit were purchased from Life Technologies (Singapore). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Singapore).

The human hepatic cancer cell line (HAK-1B) was obtained from Prof. Hirohisa Yano, Kurume University, Japan and was grown in DMEM media with 2.5% fetal bovine serum (GIBCO, Singapore) [22]. Huh-7 cells containing subgenomic HCV replicon I₃₈₉luc-ubi-neo/NS3-3/5.1 with adaptive mutation (E1202G, T1280I, K1846T) were obtained from Prof. Ralf Bartenschlager, University of Heidelberg, Germany, and were grown in DMEM media with 10% fetal bovine serum, 1 mM non-essential amino acid and 500 μ g/ml G418 (Geneticin, Merck) [23,24]. All cells were cultured in a humidified incubator at 37 °C, 5% CO₂.

2.2. Synthesis of IFN- α 2a-incorporated HA-Tyr hydrogels and rheological measurements

Hyaluronic acid-tyramine (HA-Tyr) conjugates were synthesized and the rheological measurement was performed as previously described [13]. Briefly, samples were prepared by adding 65 μ l of IFN- α 2a dissolved in PBS to 175 μ l of HA-Tyr solution (2.5 wt%), followed by addition of 5 μ l each of HRP (6.2 units/ml) and hydrogen peroxide (H₂O₂) in varying concentrations. The final concentrations of HA-Tyr, IFN- α 2a, and HRP were 1.75 wt%, 2.5×10^5 IU/ml and 0.124 units/ml, respectively. The mixture was immediately vortexed and 210 μ l was applied to the bottom plate of the rheometer. Rheological measurement was allowed to proceed until the sample's storage modulus (*G'*) reached a plateau.

2.3. Crosslinking density and mesh size of IFN- α 2a-incorporated HA-Tyr hydrogels

The mesh size (ξ) and crosslinking density (ν_e) of the HA-Tyr hydrogels were determined by previously described methods [25]. Briefly, 200 μ l of HA-Tyr hydrogels with or without IFN- α 2a were prepared and swelled in 20 ml of PBS for 24 h. The swollen hydrogels were weighed (swollen weight as M_s) and lyophilized. Then, the dried weights were determined (dried weight as M_d) and the mass swelling ratio (Q_M) was calculated by dividing M_s with M_d . The volumetric swelling ratio (Q_V), effective crosslink density (ξ) and mesh size (ν_e) were then calculated as described in the literature [25].

2.4. Release of IFN- α 2a from HA-Tyr hydrogels *in vitro*

HA-Tyr hydrogels that incorporated 2.5×10^5 IU/ml of IFN- α 2a were prepared by mixing 778 μ l of HA-Tyr conjugate (2.25 wt%) with 100 μ l of IFN- α 2a solution (2.5×10^6 IU/ml) and 112 μ l of PBS. Five microliters each of HRP and H₂O₂ (final concentrations of HRP: 0.124 units/ml and H₂O₂: 437 μ M or 728 μ M) were then added. The solution was gently mixed by pipetting and then injected between two parallel glass plates clamped 1.5 mm apart. Gelation was allowed to proceed at 37 °C for 2 h. Round gel disks, 1.6 cm in diameter, were then cut from the hydrogel slab using a circular mold. Each disk was placed in a plastic net and immersed in 20 ml of buffer solution (PBS with 0.5% BSA). At selected time points, 200 μ l of the media was withdrawn and replaced with an equal volume of fresh buffer solution to maintain a constant total volume. The collected samples were stored in LoBind tubes (Eppendorf, Germany) at 4 °C until measurement. Protein concentrations were determined using a VeriKine™ Human Interferon-Alpha ELISA kit.

2.5. Activity of IFN- α 2a incorporated in HA-Tyr hydrogels

HA-Tyr hydrogels that incorporated 2.5×10^5 IU/ml of IFN- α 2a were prepared as mentioned above. The complete degradation of the hydrogel was achieved after overnight incubation with 250 U/ml hyaluronidase at 37 °C. The solution mixture of degraded hydrogels was diluted to 2 and 4 μ g/ml IFN- α 2a with DMEM media and a Huh-7-based assay was performed on them. Typically, 200,000 of Huh-7 cells were seeded on each well of 6-well plates in DMEM with G418. After 24 h, the media was aspirated and the cells were treated with DMEM media (without G418) mixed with the solution of degraded hydrogels, or 1000 IU/ml of IFN- α 2a. After 72 h, the cell lysate was collected and a luciferase assay (Promega, Singapore) was carried out to measure the value of relative luminescence unit (RLU) for each sample. The inhibitory activity of IFN- α 2a was calculated by the following equation:

$$\text{Inhibition (\%)} = \frac{\log_{10}(\text{NC}) - \log_{10}(S_n)}{\log_{10}(\text{NC}) - \log_{10}(\text{PC})} \times 100$$

where PC is the value of RLU after incubation with 1000 U/ml IFN- α 2a, NC is the value of RLU after incubation with the negative control, and S_n is the value of RLU after incubation with samples [23].

2.6. IFN- α 2a-incorporated HA-Tyr hydrogels on inhibiting the proliferation of HAK-1B cells

Five hundred microliters of HAK-1B cell suspension containing 12,000 cells was added into each well of the 24-well plate and incubated for 2 days before treatment. HA-Tyr hydrogels with or without IFN- α 2a were prepared as described above. Fifty microliters of the hydrogel was added into each of 24-well insert chamber. After 2 h, the hydrogel-loaded inserts were placed into the wells plated with HAK-1B cells, and an additional 500 μ l of culture media was added into the insert. The whole plate was incubated for 4 days. Cell viability was assessed using alamarBlue assay according to the manufacturer's protocols. The results were expressed as percentage of viability compared with untreated cells.

2.7. Cell apoptosis assay

HA-Tyr hydrogels with 4.2×10^5 IU/ml of IFN- α 2a were prepared as described above. Two hundred microliters of gel precursor solutions of HA-Tyr conjugate, IFN- α 2a, HRP and H_2O_2 was added to each 35 mm petri dish. After 2 h, 2.5 ml of 125,000 HAK-1B cells in culture media was added to the petri dishes. As a positive control, 200 μ l of IFN- α -2a dissolved in PBS (4.2×10^5 IU/ml) was added to the petri dish with plated cells. After 4 days, the cells in each dish were trypsinized, collected and combined with the floating cells in the cell culture media. Alexa Fluor® 488 annexin V/Dead cell apoptosis kit was applied according to the manufacturer's protocol. Stained cells were sorted by flow cytometry, and the fluorescence intensities of Alexa Fluor 488 (above 1.1×10^4) and PI (above 1×10^3) were considered to be annexin V and PI positives, respectively. The results were presented as percentage of viable (annexin V negative and PI negative), apoptotic (annexin V positive and PI negative) and dead (annexin V positive and PI positive) cells.

2.8. Determination of caspase activity

An Image-iT™ live red caspase detection kit was used to detect and evaluate intracellular apoptotic events in HAK-1B cells. HAK-1B cells were plated on a glass-bottom microwell dish (MatTek Corporation, MA, USA) and treated with HA-Tyr hydrogels that incorporated 4.2×10^5 IU/ml of IFN- α -2a for 4 days. Then cells were stained with fluorescent labeled inhibitor of caspase (FLICA) and Hoechst 33342 according to protocols from Invitrogen. Confocal images were acquired with confocal laser scanning microscopy (Zeiss LSM 5 DUO).

An Apo-ONE® Homogeneous Caspase-3/7 kit was utilized to quantitatively measure caspase-3/7 activity in HAK-1B cells. After cells were treated with IFN- α 2a-incorporated HA-Tyr hydrogels for 4 days, cells were lysed with a radio immunoprecipitation assay (RIPA) buffer (Cell Signaling, USA), followed by caspase-3/7 and BCA assay according to protocols from the manufacturers. The caspase-3/7 activity of each sample (FI/ μ g) was expressed as fluorescence intensity normalized by the protein amount.

2.9. Pharmacokinetics

A mixture of 1.75 wt.% of HA-Tyr solution in PBS containing 0.125 unit/ml HRP, 6×10^7 IU/ml IFN- α 2a and different H_2O_2 concentrations (473 μ M for HA-Tyr-soft-IFN gel or 728 μ M for HA-Tyr-stiff-gel) was subcutaneously injected to the back of 6-week-old female BALB/c nude mice (Biological Resource Center, Biopolis, Singapore) based on the weight of mice (200 μ l for each weight of 20 gram). PBS solution containing same amount of IFN- α 2a was also injected as a control.

Three groups of mice ($n=4$) were treated with HA-Tyr-soft-IFN, HA-Tyr-stiff-IFN and IFN- α 2a solution, respectively. At 1, 2, 4, 8 and 24 h after the injection, 20 μ l of blood was taken from the tail vein of each mouse. The blood samples were mixed with 3 μ l of sodium citrate (37 mg/ml) to prevent blood coagulation, and then were centrifuged at 4 °C, 3,000 g for 5 min. The supernatant of each sample was then taken and stored at -20 °C before measurement. The quantity of human IFN- α 2a in the plasma of the mice was then determined by a VeriKine™ Human Interferon-Alpha ELISA kit.

2.10. Tumor regression and delivery of IFN- α 2a at tumor site

Two hundred microliters of HAK-1B cells (5×10^7 cells/ml) were subcutaneously injected to the backs of 6-week-old female BALB/c nude mice. Seven days later when diameter of the tumor reached 5–10 mm, the mice were divided into 4 groups ($n=7$) so that the average tumor size in each group was similar. Each mouse received a subcutaneous injection of 100 μ l of mixture solution of HA-Tyr conjugate (1.75 wt.%), HRP (0.124 units/ml), H_2O_2 (473 or 728 μ M) and IFN- α 2a (1.4×10^8 IU/kg) once a week for 2 weeks. Also, a subcutaneous injection of PBS and IFN- α 2a dissolved in PBS was performed once a week for 2 weeks as a comparison. Tumors were measured with a digital caliper, and the tumor volumes (mm^3) were calculated from the formula: volume = (length \times width²)/2. On day 20, the mice were sacrificed, and the tumors were then resected and fixed in 4% formalin solution.

To quantify the delivered amount of IFN- α 2a at the tumor site, three groups of mice ($n=4$) bearing HAK-1B inoculated tumors with similar size were treated with HA-Tyr-soft-IFN, HA-Tyr-stiff-IFN and IFN- α 2a solution, respectively. After 8 h of treatment, the mice were sacrificed and the tumor tissues were resected. Homogenization of tissue was performed and the supernatant of tissue lysate was taken and stored at -20 °C. The quantity of IFN- α 2a was determined by a VeriKine™ Human Interferon-Alpha ELISA kit. The care and use of laboratory animals were performed according to the approved protocols by the Institutional Animal Care and Use Committee (IACUC) at the Biological Resource Center (BRC) in Biopolis, Singapore.

2.11. Histology and immunohistochemistry

Tumor tissues were fixed in 4% formaldehyde and embedded in paraffin wax for hematoxylin and eosin staining. Primary and secondary antibodies for TUNEL (Millipore, Singapore) were used for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Primary antibody mouse anti-Ki67 NCL-Ki67-MM1 (NOVOcastra, UK) and secondary antibody anti-mouse HRP conjugate (NOVOcastra, UK) were used for Ki67 immunohistochemistry staining. Primary antibody rat anti-CD34 sc-18917 (Santa Cruz, US) and secondary antibody anti-rat HRP conjugate (Santa Cruz, US) were used for CD34 immunohistochemistry staining.

2.12. Statistical analysis

Data from *in vitro* studies are expressed as mean \pm standard deviation. Data in animal studies are expressed as mean \pm standard error of the mean (SEM). Differences between the values were assessed using one-way ANOVA and Student's *t* test, while $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Characterization of IFN- α 2a-incorporated HA-Tyr hydrogel

Previously, we reported an injectable hydrogel system composed of hyaluronic acid-tyramine (HA-Tyr) conjugates whose stiffness and gelation rate could be tuned independently by altering the concentrations