Title: Potential of alpha-fetoprotein as a prognostic marker after curative radiofrequency ablation of hepatocellular carcinoma

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Short title: Prediction of the carcinogenic potential by AFP

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Abstract

Background: Recurrence of hepatocellular carcinoma (HCC) is observed frequently, even after curative treatments. The aim of this study is to elucidate the risk factors for recurrence of HCC after radiofrequency ablation (RFA), focusing on the carcinogenic potential of the liver assessed by alpha-fetoprotein (AFP).

Method: We enrolled 357 consecutive patients who underwent complete ablation by RFA for primary HCC (≤3cm, ≤3tumors) and analyzed the correlation between 17 critical parameters, including AFP and HCC recurrence.

Results: Recurrence was observed in 236 patients during a mean observation period of 54.3 months. Multivariate analysis revealed that multiple tumors (RR=1.70, 95% CI: 1.27-2.26, p<0.001), high AFP (>10 ng/mL, RR=1.45, 95% CI: 1.09-1.94, p<0.001) and high DCP (>40 mAU/mL, RR=1.52, 95% CI: 1.13-2.02, p<0.005) were significantly correlated with recurrence. AFP was selected as a significant factor even when the cut-off level was set lower (≤5 ng/mL). The risk of recurrence increased linearly according to the increase of the lowest AFP level after RFA and the adjusted ratios relative to AFP less than 5 ng/mL were 1.56, 2.14, 2.57, and 3.13 in AFP 5-10

ng/mL, 10-20 ng/mL, 20-50 ng/mL and over 50 ng/mL, respectively. In addition, the recurrence rate was predicted by the AFP level after RFA, regardless of the level before the treatment.

Conclusions: AFP less than 5 ng/mL after curative RFA was an important predictor of a better prognosis and was considered to indicate the low carcinogenic potential of the non-cancerous liver.

Keywords: AFP, HCC recurrence, RFA

Introduction

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer-related deaths in the world. Surgical resection, liver transplantation, and radiofrequency ablation (RFA) are known as potentially curative treatment modalities[1, 2]. Recently, the increased use of screening tests has achieved detection of HCC at an early stage and the cases that can benefit from local ablation therapies have increased[3]. But the problem of recurrence remains. HCC is prone to recur and the annual rate of recurrence is reported to be approximately 20 percent, even if curative treatment was performed for the primary lesion[4, 5]. The high recurrence rate indicates that the liver was already in a hypercarcinogenic state at the time of HCC onset[6].

Serum levels of alpha-fetoprotein (AFP) are known not only as a tumor marker of the presence of HCC but also as a marker of hypercarcinogenic potential, especially for patients with chronic hepatitis C[7-12]. The risk for HCC in patients with low AFP levels (below 5 ng/mL) after a sustained virological response (SVR) was reported to be lower than that with high AFP levels[13, 14]. Moreover, Osaki et al. showed that AFP integration value was closely correlated with the risk of HCC among non-SVR patients.

The serum AFP level gradually decreases after birth to less than 10 ng/mL within 300 days and the normal adult serum AFP level is less than 20 ng/mL[15, 16]. We often set the cut-off value at 20, 100 or 200 ng/mL as a tumor marker, and many studies have used these cut-off values of AFP when examining the risk factors of HCC recurrence[9, 10, 16-19]. However, most of the studies dealing with the hypercarcinogenic potential of chronic hepatitis and/or liver cirrhosis adopted much lower cut-off values (5-10 ng/mL) and a few studies have analyzed the risk of HCC recurrence after curative treatment with these low cut-off values of AFP[8, 9, 20].

In this study, we analyzed the risk factors of HCC recurrence after treatment with RFA and tried to elucidate the potential of AFP as a marker for the hypercarcinogenic state in patients with HCC.

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Patients and Methods

Patients

We collected data from 1065 consecutive patients with newly developed hepatocellular carcinoma (HCC) between 2001 and 2013. In this study, we enrolled 357 patients with HCC who satisfied the following criteria: the tumor size was 3 cm or smaller and the number was 3 tumors or less, the Child-Pugh score was A or B, and radiofrequency ablation (RFA) was performed completely.

Informed consent was obtained from all patients. The study protocol conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki, and was approved by the ethical committee of the institute.

Diagnosis

We diagnosed HCC on the basis of typical patterns, such as an early phase hypervascular area and late phase hypovascular on dynamic computed tomography (CT) or magnetic resonance imaging (MRI).

Triple-phase dynamic CT scans were set at arterial, portal venous and

equilibrium phases. Arterial, portal and equilibrium phase scanning was started 16 sec, 60 sec and 240 sec, respectively, after injection of contrast material.

MR imaging was performed on a 1.5-T MR system and all patients who underwent MRI examinations received Gd-EOB-DTPA intravenously. Triple-phase images (arterial phase, 35 sec after arterial phase, 70 sec after arterial phase) were obtained after the injection of the contrast material. Hepatobiliary phase imaging (20 min after the administration of contrast material) was also performed.

Liver biopsy was performed when a definitive diagnosis was not provided by the imaging. The biopsy was performed percutaneously under ultrasound (US) guidance using a 21-gauge needle and histological diagnosis was performed by 2 board-certified pathologists according to the criteria prescribed by an International Working Party. Eighty nodules were histologically diagnosed as HCC.

Treatment and follow up

We performed RFA percutaneously under US guidance. We used an internally water-cooled 17-gauge cooled-tip electrode with an impedance-controlled

generator (Cool-Tip system, Valleylab, CO, USA). Dynamic CT or MRI was performed to evaluate the ablated area. When the ablated area covered an entire nodule, we defined the status as “complete necrosis.” RFA was repeated until complete necrosis of HCC was confirmed. The median number of RFA session was 1 (range:1-5) and the number of SAE was 18, including 3 sustained pleural effusion, 2 ectopic ablation, 2 hepatic infarction and 2 bile duct dilatation.

The patients underwent blood tests, including AFP, 1 month after RFA, followed by further tests every month or two, US every 2 or 3 months, in addition to the CT or MRI examinations at least twice a year. The evaluation for recurrence was made using the same diagnostic criteria as for primary lesions. Local recurrence was diagnosed via the emergence of a tumor in contact with the primary lesion and intrahepatic distant recurrence was diagnosed by the occurrence of a new HCC in the liver that did not meet the criteria for local recurrence.

Statistical analysis

AFP, *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3) and,

des-γ-carboxy prothrombin (DCP) levels before RFA, age, sex, hepatitis B surface antigen (HBsAg), hepatitis C virus antibody (anti-HCV), alcohol drinking (>70 g/day), Child-Pugh score, tumor size, tumor number, total bilirubin (T-bil), albumin (Alb), aspartate aminotransferase (AST), alanine aminotransferase (ALT), platelets (Plt), prothrombin time (PT) were initially examined as variables correlated with HCC recurrence using the Cox Proportional Hazard model. The lowest level of AFP, AFP-L3, and DCP after RFA were also analyzed as alternatives to these markers prior to the treatment. Moreover, we calculated the adjusted risk of HCC recurrence at different pre-treatment AFP levels and post-treatment AFP levels. We corrected the risk by sex, albumin, AST, PLT, size of tumor, number of tumors, and DCP. The Kaplan-Meier method was used to determine the cumulative recurrence rate and differences were evaluated by the log-rank test. All statistical analyses were performed using JMP software, version 11.0 (SAS Institute, Inc., Cary, NC, USA). P-values smaller than 0.05 were considered significant.

Results

The patient profiles at baseline and post-RFA are shown in Table 1. Among the 357 patients in this study, 222 were male (62.7%) and their mean age was 73 years. Forty-three patients had HBV infection, 289 had HCV infection, and 29 had no hepatitis virus infection. Four had both HBV and HCV infection. Most of the patients (n=300, 84.0%) were classified to Child-Pugh A. The number of patients who received interferon, DAA, nucleoside analogue, and BCAA was 271, 0, 23, and 33, respectively. The average observation period was 54.3 months. Two hundred and thirty six patients had a recurrence of HCC during the observation period. Extrahepatic recurrence was observed in one case (bone metastasis).

Risk for the recurrence of HCC after RFA

Univariate analysis showed that male sex, low albumin and platelet count, high aspartate aminotransferase (AST, >40 IU/L), AFP (>10 mg/mL) and des-γ-carboxyprothrombin (DCP, >40 mAU/mL), large tumor (>2 cm), and multiple tumors were significantly correlated with a rate of high HCC recurrence (Table 2).

Multivariate analysis of the significant factors in univariate analysis revealed that multiple tumors (HR=1.70, p<0.001), high AFP (>10 ng/mL, HR=1.45, p=0.001) and high DCP (>40 mAU/mL, RR=1.52, p=0.005) were significant risk factors for recurrence. The same analysis was performed using AFP and DCP after RFA, instead of before RFA. The two tumor markers after RFA were_also significant factors for recurrence.

The effect of AFP level for the recurrence of HCC

We examined the recurrence-free survival of patients with different levels of AFP. The serum AFP levels before RFA were closely correlated with survival. The 3 year recurrence-free survival rates in patients with AFP levels before RFA less than 5 ng/mL, 5-10 ng/mL, 10-20 ng/mL and more than 20 ng/mL were 57.7%, 37.2%, 26.2% and 30.8%, respectively (p=0.001). A similar correlation was observed between the recurrence rate and AFP after RFA. The 3 year recurrence free survival rates were 52.8%, 34.5%, 26.3% and 19.9% in patients with AFP less than 5 ng/mL, 5-10 ng/mL, 10-20 ng/mL and more than 20 ng/mL, respectively (p<0.001). A clear difference was

observed between patients with AFP less than 5 ng/mL and AFP 5-10 ng/mL (p=0.003) . Most of the recurrences were intra-hepatic distant recurrences (199/232, 86.1%) and local recurrence was observed only in 33 patients. While intra-hepatic distant recurrences were closely correlated with AFP before RFA as well as AFP after RFA, no correlation was observed between local recurrence rate and AFP before RFA (Supplemental Figure 1-4). The local recurrence rate in patients with high AFP (10ng/mL) after RFA was slightly higher than that with low AFP (p=0.04; log-lank test). We also examined the effect of DCP on recurrence free survival. The 3 year recurrence free survival rates were 34.3%, 42.5%, 32.2% and 17.6% in patients with DCP less than 10 mAU/mL, 10-20 mAU/mL, 20-40 mAU/mL and more than 40 mAU/mL, respectively (p=0.002) (Fig.1).

Adjusted risk for recurrence in patients with different AFP levels

The adjusted risk of HCC recurrence was calculated for different pre-treatment and post-treatment AFP levels. The risk increased according to the elevation of AFP before RFA, except for AFP over 50 ng/mL, while a clear linear correlation was

observed between the risk and AFP after RFA (Fig.2). The relative risk to AFP less than 5 ng/mL before RFA were 1.41 (p=0.12), 1.69 (p=0.03), 2.11 (p=0.001), and 1.67 (p=0.03) for AFP 5-10 ng/mL, 10-20 ng/mL, 20-50 ng/mL and over 50 ng/mL, respectively. The relative risk to AFP less than 5 ng/mL after RFA were 1.56 (p=0.021), 2.14 (p<0.001), 2.57 (p<0.001), and 3.13 (p<0.001) for AFP 5-10 ng/mL, 10-20 ng/mL, 20-50 ng/mL and over 50 ng/mL, respectively.

Relationship between the recurrence free period and AFP after RFA

The lower the AFP levels were after RFA, the lower the risk of recurrence after RFA was. The highest levels of the minimum AFP after RFA were 409.3 ng/mL, 268.9 and 23.0 ng/mL in patients with recurrence within 3 years, 3-5 years and 5-7 years, respectively. The level in patients without recurrence at 7 years was 11.2 ng/mL (Fig. 3).

The effect of normalization of AFP after RFA

The AFP levels frequently decreased to normal after RFA, so we examined the

effect of normalization of AFP. If the AFP levels were normal (<5 ng/mL) after RFA, even in cases with high AFP levels before RFA, the recurrence rate was as low as for the patients with normal AFP levels before RFA. Even if the cut-off values of AFP were changed to 10 or 20 ng/mL, the same relationships were observed (Fig.4).

Discussion

In this study, multivariate analysis revealed that AFP and DCP levels, before and after treatment, and the tumor number were significantly correlated with HCC recurrence after RFA. The serum AFP values were related to the prognosis, even when the cut-off levels were set lower. In particular, AFP less than 5 ng/mL after treatment is an important indicator of a better prognosis. The risk of HCC recurrence increased linearly according to the elevation of the minimum AFP levels after treatment. Conversely, there were no cases who achieved long-term recurrence-free survival if the AFP level after treatment was high. However, low AFP levels did not always guarantee long-term recurrence-free survival. It is necessary for long-term survival that the AFP level after treatment is low.

AFP is an oncofetal protein produced by fetal hepatocytes, gastrointestinal cells and yolk-sac cells[15, 21]. The colloid osmotic pressure is controlled by serum AFP in embryonic development and by albumin after birth. Then, serum AFP levels decrease gradually to less than 10 ng/mL within 200-300 days[16, 21]. The normal AFP level is <20 ng/mL in the blood of a healthy adult. AFP is known to be produced by

HCC cells that are in a dedifferentiated state compared to normal hepatocytes[15, 22]. Thus, even if there is no obvious cancer, elevation of serum AFP levels is considered to suggest that hepatocytes have begun de-differentiation and development into a carcinogenic state. The elevation of serum AFP levels after RFA means not only that HCC still remains, but also that the non-cancerous liver is in a highly carcinogenic condition.

Although a lot of studies have shown that the risk of HCC recurrence after RFA was associated with the serum AFP levels, almost all such studies have treated AFP as a tumor marker[9, 16, 17, 23]. Thus, many previous reports have used a high AFP cut-off value (20-200 ng/mL)[24-26]. In our study, we focused on AFP as a carcinogenic marker and examined its potential at a low cut-off value, such as 5 ng/mL, which was used to express the carcinogenic potential of chronic hepatitis C patients after SVR[14]. Some other studies have investigated the role of AFP using a low cut-off value. Kudo et al. suggested that a low AFP level (<10 ng/ml) before hepatectomy had a high predictive value for a low rate of recurrence and long-term survival after the treatment, however the study dealt with only the serum AFP levels before treatment[27]. Witjes et al. used a

single cut-off level and reported that high AFP levels (>9 ng/mL) after surgical resection were significantly associated with the clinical outcome of HCC patients without well-established risk factors[19]. In this study, we paid attention to the serum AFP levels before and after treatment, and compared the risk using various cut-off levels.

To minimize the effect of the residual tumor on the AFP level, here, we investigated HCCs less than 3 cm in size and fewer than three in number. We also conducted the same analysis with single small HCCs less than 2 cm in diameter and the same correlation was seen between AFP after RFA and the recurrence of HCC (data not shown). In addition, the correlation was observed mainly in intra-hepatic distant recurrence. Moreover, the rate of recurrence in cases with normal AFP after RFA but high AFP before treatment was the same as that in cases with normal AFP before treatment. These result indicated that the levels of AFP after RFA in this study were mainly determined by the production of AFP by non-cancerous hepatocytes, as we observed in the SVR patients[14].

DCP is another good tumor marker for HCC[24, 28-30]. Recurrence free survival was short in patients with high DCP (>40 mAU/mL) after RFA. A high DCP

after RFA might indicate the presence of residual tumor tissue; however, over 90% of the patients had a low DCP level (less than 40 mAU/mL) and no correlation between DCP and recurrence free survival was observed, especially in patients with a single small HCC less than 2 cm in diameter (data not shown). Because its low discriminatory ability, DCP does not seem to be a marker of the hyper-carcinogenic state. AFP-L3 might be the other marker for knowing the carcinogenic potential; however, we could not examine the ability because of the number of patients who were positive for AFP-L3 after RFA was small (n= 7).

Our study has some limitations. The major limitation is its retrospective and non-multicenter design. More large scale and prospective studies will confirm our outcomes for predicting carcinogenesis.

In conclusion, serum AFP levels before and after treatment were significant predictive factors of HCC recurrence, even when the AFP cut-off level was set lower, and the level after curative RFA treatment indicated the carcinogenic potential of non-cancerous hepatocytes.

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Table 1. Patient Characteristics

Variables		
Sex (male)	222	(62.7%)
Age, years	73	(40-93)
HBV/HCV	43/289	(12.1%/81.4%)
Child-Pugh: A	300	(84.0%)
Tumor size, mm	16	(7-30)
Tumor number (solitary)	245	(68.6%)
AFP, ng/ml	11.8	(0.6-2818)
DCP, mAU/ml	25	(9-9491)
AFP post RFA, ng/ml	7.4	(0.9-409.3)
DCP post RFA, mAU/ml	19	(3.8-940)

All numbers are the median (range) unless otherwise stated.

HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, alpha-fetoprotein; DCP, des-γ-carboxy prothrombin; AFP-L3, *Lens culinaris* agglutinin-reactive fraction of AFP; RFA, radiofrequency ablation.

Table 2. Predictiors of recurrence after RFA

	univariate analysis			multivariate analysis		
Variables	HR	95%CI	P-value	HR	95%CI	P-value
sex (male)	1.35	1.03-1.77	0.026	1.19	0.90-1.58	0.214
Age, (>70yr)	1.13	0.87-1.47	0.331			
anti-HCV positive	1.2	0.87-1.47	0.261			
HBsAg positive	0.91	0.60-1.34	0.674			
Alcohol (>70g/day)	0.88	0.57-1.32	0.889			
Child-Pugh A	0.99	0.66-1.42	0.968			
T.Bil (>1mg/dL)	1.24	0.94-1.62	0.125			
Alb (>3.5g/dL)	0.69	0.53-0.90	0.007	0.77	0.57-1.05	0.101
AST (＞40IU/L)	1.35	1.02-1.82	0.032	1.07	0.78-1.48	0.666
ALT (＞40IU/L)	1.22	0.94-1.58	0.131			
Plt (＞$10\times10^4/\mu L$)	0.75	0.58-0.97	0.03	0.89	0.66-1.21	0.465
PT (＞80%)	1.02	0.75-1.41	0.867			

Tumor size (＞20mm)	1.65	1.23-2.19	0.001	1.24	0.89-1.70	0.195
Tumor number (multiple)	0.59	0.45-0.78	<0.001	1.7	1.27-2.26	<0.001
AFP (＞10ng/mL)	1.69	1.30-2.21	<0.001	1.45	1.09-1.95	0.01
DCP (＞40mAU/mL)	1.6	1.21-2.10	<0.001	1.52	1.14-2.02	0.005
AFP post RFA (＞10ng/mL)	1.94	1.49-2.53	<0.001	1.86	1.39-2.49	<0.001
DCP post RFA (＞40mAU/mL)	1.97	1.30-2.88	0.001	2.18	1.42-3.24	<0.001

† Only variables that had a P-value <0.05 in univariate analysis were entered into the multivariate cox proportional hazard model. The hazard ratios (HR) of AFP/DCP before and after RFA were examined separately in multivariate analysis.

CI, confidence interval.

Figure legends

Figure 1. The recurrence-free survival rate in HCC patients with different AFP levels before RFA (A) and after RFA (B). The recurrence-free survival of patients with low AFP levels was significantly shorter than of those with high AFP levels in both cases ($p<0.001$; log-rank test). The recurrence-free survival rate in HCC patients with different DCP levels after RFA (C).

Figure 2. The adjusted risk of recurrence in patients with different AFP levels before and after RFA. The risk increased linearly according to the increase of AFP except for AFP over 50 ng/mL before RFA. The risk was corrected by sex, albumin, AST, PLT, size of tumor, number of tumors, and DCP. Bars indicated 95% confidence interval.

Figure 3. Relationship between the recurrence free period and AFP after RFA. The highest levels of the minimum AFP after RFA were 409.3, 268.9 and 23.0 ng/mL in patients with recurrence within 3 years, 3-5 years and 5-7 years, respectively. The level in patients without recurrence at 7 years was 11.2 ng/mL.

Figure 4. The effect of normalization of AFP on recurrence-free survival. No significant difference in recurrence was observed between patients with normal AFP before treatment (coarse dotted line) and with abnormal AFP before treatment but normalized after RFA (fine dotted line). Patients with abnormal AFP after RFA (solid line) showed worse recurrence free survival, regardless of the AFP cut-off levels ($p<0.001$; log-rank test）. AFP cut-off levels were 5 ng/ml, 10 ng/ml and 20 ng/ml in (A), (B) and (C), respectively.

Titles for supplemental figures

Supplemental figure 1. The distant recurrence rate in HCC patients with different AFP levels before RFA.

Supplemental figure 2. The distant recurrence rate in HCC patients with different AFP levels after RFA.

Supplemental figure 3. The local recurrence rate in HCC patients with different AFP levels before RFA.

Supplemental figure 4. The local recurrence rate in HCC patients with different AFP levels after RFA.