Featured Article

Phase II Randomized Trial of Autologous Formalin-Fixed Tumor Vaccine for Postsurgical Recurrence of Hepatocellular Carcinoma

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Abstract

Purpose: **We conducted a Phase II clinical trial with randomized patients to determine whether autologous formalin-fixed tumor vaccine (AFTV) protects against postsurgical recurrence of hepatocellular carcinoma (HCC).**

Experimental Design: **Forty-one patients with HCC who had undergone curative resection were randomly allocated to the vaccine treatment ($n = 19$) or no adjuvant control group ($n = 22$). Three intradermal vaccinations were administered at 2-week intervals beginning 4–6 weeks after hepatic resection. A delayed-type hypersensitivity test was performed before and after vaccination. Primary and secondary end points are recurrence-free survival and overall survival, respectively. Observation continued until the majority of surviving patients had lived >12 months after the curative resection.**

Results: **In a median follow-up of 15 months, the risk of recurrence in vaccinated patients was reduced by 81% (95% confidence interval, 33–95%; $P = 0.003$). Vaccination significantly prolonged the time to first recurrence ($P = 0.003$) and improved recurrence-free survival ($P = 0.003$) and overall survival rates ($P = 0.01$). AFTV played a significant role in preventing recurrence in patients with small tumors. Adverse effects were limited to grade 1 or 2 skin toxicities such as erythema, dry desquamation, and pruritus.**

Conclusions: **AFTV therapy is a safe, feasible, and effective treatment for preventing postoperational recurrence of HCC. Patients with low tumor burdens benefit from the treatment. This treatment should be advanced to a large-scale randomized trial.**

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, and its incidence is increasing (1, 2). Vaccination with hepatitis B surface antigen and IFN therapy for hepatitis C may reduce the incidence of HCC in the future (3, 4), but effective treatment for HCC in hepatitis-prevalent countries such as China, where one-third of new cases arise (5, 6), is urgently needed. Curative treatments such as hepatic resection, orthotopic liver transplantation, or percutaneous regional treatments offer the only chance of cure, but most cases are ineligible at diagnosis due to advanced tumor stage or poor liver function (6–14). Furthermore, the high frequency of postoperative recurrence decreases the long-term survival rate (5–12).

Recurrence control is the primary goal of novel HCC treatments. However, an efficient treatment for reduction of HCC recurrence has not yet been governmentally approved. Active specific immunotherapy using the patient's own tumor to elicit a long-term cell-mediated immune response has been used successfully in treatment of melanoma, renal carcinoma, and colon cancer (15–17). We developed a HCC vaccine consisting of autologous formalin-fixed tumor tissue fragments, biodegradable microparticles containing human granulocyte macrophage colony-stimulating factor and human interleukin 2, and tuberculin (18). In a nonrandomized Phase I/II clinical trial, this vaccine caused few adverse effects and significantly improved the recurrence-free survival of patients who had undergone hepatectomy, compared with historical controls. The efficacy of autologous formalin-fixed tumor vaccine (AFTV) in the control of postsurgical recurrence of HCC was more precisely evaluated in the randomized Phase II clinical trial reported here.

Patients and Methods

Patients admitted to the Department of Hepatobiliary Surgery, First Affiliated Hospital of Sun Yat-sen University were enrolled in this study. Diagnosis of HCC was histologically confirmed before randomization. Other eligibility criteria included the International Union Against Cancer (1997, 5th edition) clinical tumor-node-metastasis (TNM) groupings of stage I, II, or IIIA; curative resection; Child-Pugh class A or B hepatic function; adequate bone marrow and renal reserves [leukocytes $> 3{,}000/\mu l$, platelets $> 100{,}000/\mu l$, serum creatinine $<1.5\times$ the upper limits of normal]; Eastern Cooperative Oncology Group performance status < 2 (Karnofsky $> 60\%$); age 18–80 years; and signed written informed consent. Exclusion criteria included TNM stage IIIB, IVA, or IVB; existing second malignancy or history of second malignancy in the past 5 years; uncontrolled medical problems; postoperative dysfunction of vital organs; or systemic steroid therapy, chemotherapy, radiotherapy, or other immunotherapy within 1 month of the study entry.

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Study Design. Hepatectomies were performed by experienced surgeons at the participating hospital. Curative resection was defined as the macroscopically complete removal of the tumor and a histologically proven tumor-free margin. Other microscopic criteria included encapsulation of resected tumors, vascular invasion or intrahepatic metastasis, and Edmondson's grading of tumor cell differentiation. During the operations, sufficient tumor tissues were taken from the excised samples and fixed immediately in 10% neutral formalin. Within 1 week, eligible patients were randomly allocated to the AFTV therapy group or no adjuvant control group. Randomization using permuted blocks without stratification and allocation were performed by a person not otherwise associated with this study. The formalin-fixed HCC tissues were sent to the laboratory of RIKEN Cell Bank in Japan for vaccine preparation. Ethical approval from the hospital's medical review boards and written informed consent from all enrolled patients were obtained.

From the results of our previous clinical trial (18), we have set primary and secondary end points to be recurrence-free survival and overall survival, respectively. Intermediate analysis was preset at a time when the majority of surviving patients in both the control and vaccine groups had survived >12 months after curative resection because adjuvant therapy with AFTV has revealed, when compared with the latest historical control, a large reduction of the recurrence rate essentially by 12 months after curative resection (see Fig. 2*A* of Ref. 18). Pretrial assumption of the number of required patients (n) was made as follows, according to Ref. 19.

$$n = [e/(2 - S_{1(12month)} - S_{0(12month)}]/(1 - w)$$

$$e = [(\theta + 1)/(\theta - 1)]^2(Z_{\alpha/2} + Z_{\beta})^2$$

$$\theta = \log(S_{1(12month)})/\log(S_{0(12month)})$$

where,

$S_{1(12month)}$ is the rate of recurrence-free survival of the vaccine group at 12 months (we set this value at 0.7, assumed from Fig. 2*A* shown in Ref. 18),

$S_{0(12month)}$ is the rate of recurrence-free survival of the control group at 12 months (this value was 0.375 from Fig. 2*A* shown in Ref. 18),

w represents the rate of drop-outs, and

$Z_{\alpha/2}+Z_{\beta} = 2.801$ (from the table of normal distribution; we have set the significance level at 5% and the power of the test at 80%).

When we set possible drop-outs to be 25% ($w = 0.25$) because many ineligible patients were found in the prior study, n was calculated to be 52. Two more patients were added when we observed that one patient allocated to control group was found ineligible, and one patient allocated to the vaccine group refused the adjuvant therapy.

The preparation of AFTV has been described previously (18). Briefly, formalin-fixed HCC tissue (2–3 g wet weight) was fragmented by homogenization and filtered through 70-μm nylon meshes, sterilized with 70% alcohol, washed with 0.9% saline, and incubated in RPMI 1640 at 37°C for 2 days to inactivate residual formalin. The fragments were washed with 0.9% saline and packed by microcentrifugation at 15,000 rpm for 5 min. HCC fragments quantified by packed-volume, human granulocyte macrophage colony-stimulating factor microparticles, human interleukin 2 microparticles, and tuberculin were preserved at 4°C and mixed just before bedside administration. Microbial contamination was avoided by use of sterile procedures.

AFTV was administered 4–6 weeks after hepatectomy, so that the patients could recover sufficiently from any immunosuppression that may have been induced by anesthesia and surgery. Eligible patients received three intradermal vaccinations at 2-week intervals. The treatment dose [dose 4 in the previous work (18)] included 40 μl of packed autologous formalin-fixed HCC fragments, 4000 units of human interleukin 2 microparticles and 4000 units of human granulocyte macrophage colony-stimulating factor microparticles (provided by the Department of Biomedical Engineering, School of Medicine, Johns Hopkins University, Baltimore, MD); 50 ng of tuberculin (BCG Inc., Tokyo, Japan); and 60 μl of 0.9% saline. Each vaccination consisted of five intradermal injections of 0.1 ml of dose 4 (0.5 ml of vaccine in total) into the upper arm at five different sites; three vaccinations completed the treatment. A delayed-type hypersensitivity skin test, performed 48 h before the first vaccination and 2 weeks after the third vaccination, consisted of intradermal injection into the forearm of a suspension of 10 μl of packed autologous fixed HCC fragments and 90 μl of 0.9% saline. A positive delayed-type hypersensitivity response was defined as the occurrence of erythema and induration at the local site 48 h after the injection, with a longest diameter of 10 mm or more.

After the first vaccination, all adverse events were documented. We assessed severity according to the WHO grading system of acute and subacute toxicities. Postoperative follow-up of patients in both groups included hepatic ultrasonography and determination of serum α-fetoprotein levels every 2 months, a computed tomography scan every 6 months or any time suspected nodules were found by ultrasonography, and chest radiography every 6 months. Pre- and post-vaccination liver function tests were performed to assess the possible impairment of liver function induced by AFTV. Treatment would be terminated on observation of vaccination-related grade 3 or 4 toxicities, autoimmune diseases, or HCC recurrence. These patients would be immediately withdrawn from the study and subjected to other HCC treatments.

Recurrence was defined as the presence of new intrahepatic lesions identified by imaging studies and histological confirmation by biopsy. All of the first recurrences and deaths were documented. Follow-up of patients with one or more recurrences continued, but only the period between hepatectomy and the first recurrence was used for statistical analysis. Patients with HCC recurrence in this study received a second hepatectomy, percutaneous radiofrequency ablation, or transcatheter chemoembolization.

Statistical Analysis. We used StatView software (version 5.0; SAS Institute, Inc.) for statistical analysis. We generated Kaplan-Meier curves for time to first recurrence, recurrence-free survival, and overall survival and compared the two groups by log-rank statistics. The Cox proportional hazards model was used for estimation of risk reduction. Univariate analysis (log-rank test) was used to assess the prognostic relevance of AFTV therapy and baseline characteristics to recur-

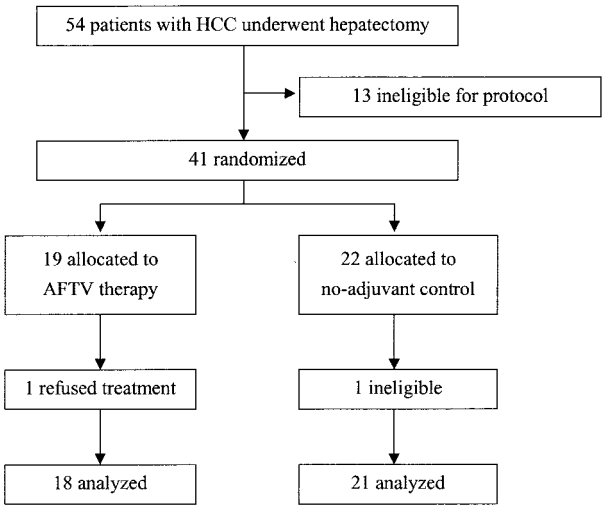

Fig. 1 Trial profile.

rence and survival. Variables with $P < 0.05$ were used in Cox proportional hazards model for multivariate analysis. All statistical tests were two-sided, and the significance level was set at $P < 0.05$.

Results

From January 2001 to January 2003, we performed hepatectomies on 54 patients with a preoperative diagnosis of HCC. Thirteen patients were excluded from the study: five for noncurative resection; four for stage IVA or IVB disease; two for postoperative liver failure; and two because they preferred transcatheter chemoembolization after operation. Forty-one patients were enrolled, but one patient was found to be ineligible after randomization because cancer cells were detected in pleural effusion 3 weeks after the operation, and one patient refused to receive the vaccination (Fig. 1). The baseline characteristics of the patients in the two groups are listed in Table 1. The median follow-up time for all patients was 15 months (range, 8–28 months).

In the vaccine group, erythema, dry desquamation, and pruritus at the vaccinated sites were observed after each vaccination. These painless grade 1 and 2 toxicities resolved within 2 weeks without medical intervention. Grade 3 or 4 toxicities such as moist desquamation, ulceration, or necrosis were not observed. Lymphadenopathy and systemic reactions such as fever or chills did not occur in any case. Vaccination-related impairment of vital organ (*i.e.*, liver, kidney, and bone marrow) functions was not found. Occurrence of autoimmune disease was not observed. None of the patients complained about the skin toxicities caused by vaccination or withdrew from the study because of adverse effects.

Three (17%) vaccinated patients and 13 (62%) nontreated control patients had confirmed recurrence of HCC (Table 2). Six (46%) solitary recurrences, five (38%) multiple recurrences, one (8%) distant metastasis, and one (8%) multicentric occurrence were observed in the control group; one (33%) solitary recurrence and two (67%) multiple recurrences were observed in the AFTV group (Table 3). The median time from hepatectomy to the first recurrence was 6.6 months (range, 2–13 months) in the control group and 10.3 months (range, 9–11 months) in the vaccine group. Compared with the control group, AFTV decreased the frequency of overall recurrence by 45% and reduced the risk of recurrence by 81% (95% confidence interval, 33–95%; $P = 0.01$; Table 2). In addition, the time to first recurrence in the vaccine group was much longer than that in the control group ($P = 0.003$; Fig. 2). AFTV also significantly improved recurrence-free survival compared with the no adjuvant con-

Table 1 Patient characteristics

	Vaccine ($n = 18$)	Control ($n = 21$)
Sex		
Male	16 (89%)	19 (90%)
Female	2 (11%)	2 (10%)
Age		
Average ± SD	48 ± 9 yrs	47 ± 13 yrs
<60 yrs	15 (83%)	17 (81%)
≥60 yrs	3 (17%)	4 (19%)
Cause of liver injury		
Hepatitis B	17 (94%)	18 (85%)
Hepatitis C	0 (0%)	1 (5%)
Unknown	1 (6%)	2 (10%)
Alanine aminotransferase (IU/liter)		
<100	18 (100%)	16 (76%)
≥100	0 (0%)	5 (24%)
Child-Pugh class		
A	17 (94%)	19 (90%)
B	1 (6%)	2 (10%)
Background liver		
Chronic hepatitis	7 (39%)	8 (38%)
Cirrhosis	10 (55%)	11 (52%)
Unknown	1 (6%)	2 (10%)
AFP[a] (ng/ml)		
<400	8 (44%)	12 (57%)
≥400	10 (56%)	9 (43%)
Tumor number		
Single	17 (94%)	20 (95%)
Multiple	1 (6%)	1 (5%)
Tumor size (mm)		
Average ± SD	54 ± 28	53 ± 32
<50	9 (50%)	10 (48%)
≥50[b]	9 (50%)	11 (52%)
Tumor spread[c]		
Yes	6 (33%)	7 (33%)
No	12 (67%)	14 (67%)
AJCC stage		
I or II	11 (61%)	13 (62%)
IIIA	7 (39%)	8 (38%)
Hepatectomy[d]		
Minor	11 (61%)	13 (62%)
Major	7 (39%)	8 (38%)
Edmondson's grade		
1 or 2	15 (83%)	19 (90%)
3	3 (17%)	2 (10%)

[a] AFP, α-fetoprotein; AJCC, American Joint Committee on Cancer.

[b] Included multiple tumors.

[c] Tumor spread was defined as histological proof of regional infiltration through capsule or vascular invasion.

[d] Minor hepatectomy was limited tumor resection or subsegmentectomy; major hepatectomy was segmentectomy or lobectomy.

Table 2 Recurrence in different patient groups

Patient group	Vaccine	Control	P^a	Hazard ratio (95% CI)[b]	P^c
All patients	3/18 (17%)	13/21 (62%)	0.003	0.19 (0.05–0.67)	0.01
Tumor size ≥50 mm[d]	3/9 (33%)	10/11 (91%)	0.003	0.19 (0.05–0.69)	0.01

[a] Log-rank test.
[b] CI, confidence interval.
[c] Wald test.
[d] Included multiple tumors.

Table 3 Recurrence and death

Patient no.	Recurrence type	Prognosis
Control-2	Bone metastases	Died of disease
Control-3	Solitary	Died of disease
Control-4	Multiple, portal vein thrombi	Died of disease
Control-5	Solitary, multicentric occurrence[a]	Died of disease
Control-7	Solitary	Died of disease
Control-10	Solitary	Alive
Control-12	Multiple, portal vein thrombi	Died of disease
Control-13	Multiple, portal vein thrombi	Died of disease
Control-14	Solitary	Alive
Control-15	Multiple	Died of disease
Control-16	Solitary	Alive
Control-17	Multiple	Alive
Control-21	Solitary	Alive
Vaccine-5	Multiple	Alive
Vaccine-8[b]	Multiple, portal vein thrombi	Died of disease
Vaccine-9	Solitary	Alive

[a] The new lesion was Edmondson's grade 1.
[b] Delayed-type hypersensitivity response positive.

trol ($P = 0.003$). Although precise analyses for subset of patients have not necessarily been planned in advance, no recurrence (0 of 9 patients) was observed in vaccinated patients with tumors <50 mm in size, whereas recurrence was found in 30% (3 of 10 patients) of the control group. AFTV therapy significantly improved recurrence-free outcomes in patients with tumors ≥50 mm in size ($P = 0.003$).

Of all 14 variables (Table 1) assessed by univariate analysis, only AFTV therapy and tumor size were significantly related to recurrence (Table 4). Multivariate analysis revealed that the risk of recurrence in the vaccine group remained unchanged compared with the control group. There was a significant interaction between recurrence after vaccination and tumor size ($P = 0.0004$), but the effect was clinically important because all recurrences in the vaccine group were observed in patients with tumors ≥50 mm in size (three of nine patients, 33%), whereas all nine vaccinated patients with tumors <50 mm in size were recurrence free.

A positive delayed-type hypersensitivity response was confirmed in 12 patients (12 of 18, 67%) with a mean erythematic area of 11.9 mm. With the exception of one patient with multiple recurrences (Table 3, patient Vaccine-8), 92% (11 of 12 patients) of the patients were recurrence free at the end of the observation, whereas recurrence occurred in 33% (2 of 6) of the vaccinated patients with a negative delayed-type hypersensitivity response.

Overall survival differed significantly between the vaccine and control groups ($P = 0.01$; Fig. 3). AFTV therapy improved overall survival by 89% (95% confidence interval, 9–99%; $P = 0.01$). The survival rate of vaccinated patients with tumors <50 mm in size, TNM stage I/II disease, or negative invasive histology was 100% (0 of 9, 0 of 11, and 0 of 12, respectively), compared with 20% (2 of 10), 23% (3 of 13), or 31% (4 of 13) in the control group. Overall survival in patients with tumors ≥50 mm in size was also significantly improved ($P = 0.04$). At the end of the follow-up, the mortality rate was 6% (1 of 18) in the vaccine group and 38% (8 of 21) in the control group. All deaths were related to HCC recurrence. Multiple recurrences contributed to four of the deaths (50%) in the control group and one death (100%) in the vaccine group (Table 3). Solitary recurrence, distant metastasis, and multicentric occurrence were responsible, respectively, for two deaths (25%), one death (13%), and one death (13%) in the control group.

Discussion

AFTV therapy significantly lowered the incidence of early recurrence of HCC in patients who had undergone hepatic resection. Hepatectomy reduces the tumor burden, and most novel adjuvant approaches are developed to prevent postsurgical recurrence. Randomized clinical trials evaluating the efficacy of adoptive immunotherapy, acyclic retinoid, intra-arterial ^{131}I-labeled lipiodol, and IFN-α have recently shown promise (20–23). However, immediate clinical application of these ap-

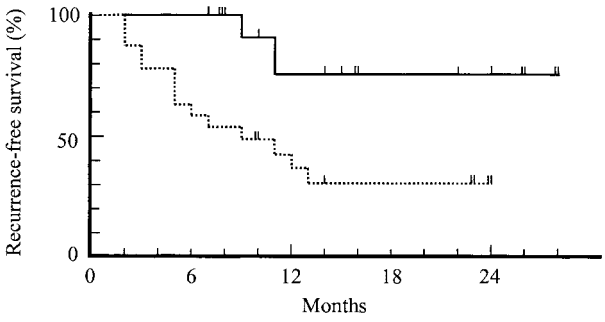

Fig. 2 Recurrence-free survival. $n = 21$ for control (*dotted line*). $n = 18$ for autologous formalin-fixed tumor vaccine (*solid line*). $P = 0.003$ (log-rank test).

Table 4 Analyses of overall recurrence

Variable	Univariate analysis P^a	Multivariate analysis Hazard ratio (95% CI)[b]	Multivariate analysis P^c
AFTV therapy (yes *vs.* no)	0.003	0.14 (0.04–0.52)	0.003
Tumor size (<50 mm *vs.* ≥50 mm)	0.004	0.15 (0.04–0.54)	0.004

[a] Log-rank test.
[b] CI, confidence interval; AFTV, autologous formalin-fixed tumor vaccine.
[c] Wald test.

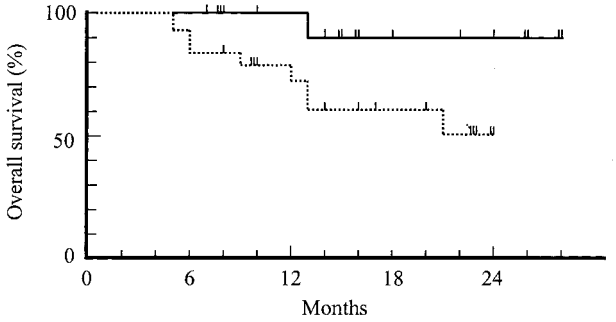

Fig. 3 Overall survival. $n = 21$ for control (*dotted line*). $n = 18$ for autologous formalin-fixed tumor vaccine (*solid line*). $P = 0.01$ (log-rank test).

proaches is limited by *ex vivo* preparation of effector cells, the high cost of the drugs, systemic adverse effects, or incompatibility with hepatitis C.

Active immunotherapy can induce tumor-specific CTLs and achieve a long-term antitumor immune response (15–17). The HCC-associated antigen α-fetoprotein serves as a target for T-cell immunotherapy in animals (24, 25), but only patients carrying matched MHC alleles benefit from tumor-associated antigen-based vaccine therapy (26). Although antigen-preloaded dendritic cells elicit a strong antitumor immune response (27, 28), dendritic cell-based approaches are too cumbersome and costly for use in large-scale clinical trials. Until HCC-specific antigens are fully identified, the tumor cell itself is still the best source of tumor antigen. We successfully induced tumor-specific CTLs from the peripheral blood of tumor-bearing patients using autologous formalin-fixed paraffin-embedded tumor sections (29). The autologous fixed tumor tissue is expected to provide many tumor antigens that may be recognized by a patient's immune system to induce a specific antitumor response. Local use of human granulocyte macrophage colony-stimulating factor activates dermal antigen-presenting cells, whereas human interleukin 2 expands the proliferation of induced CTLs, and the induced antitumor immune response is amplified. Release of these cytokines from microparticles maintains long-term stimulation of the immune system and may induce persistent antitumor immunity.

Because most patients enrolled in this trial had a history of hepatitis B, these results are useful as preliminary safety and efficacy data for a randomized clinical trial to evaluate this stimulatory treatment for HCC in hepatitis B-endemic countries. The baseline characteristics of the two groups were well balanced, and the incidence of recurrence in the control group was similar to that in our previous report (18). Our results also indicate that recurrence is the main cause of death in patients with HCC. One of the two factors that reduced the risk of recurrence in this study was AFTV therapy, indicating that this vaccine may successfully induce antitumor immunity against micrometastatic tumors. Tumor size is another important factor that influences postsurgical recurrence of HCC. Time to first recurrence differed significantly between control patients with tumors of $\geq$50 mm and $<$50 mm in size ($P = 0.003$). Patients with small tumors, low TNM stages, or negative invasive histology benefit the most from vaccination. Moreover, AFTV significantly reduced recurrence risk and improved survival outcomes in patients with large tumors. Large tumors are considered common factors of early recurrence and poor prognosis. These findings demonstrate that the anti-HCC immunity induced by AFTV is relatively potent and persistent and most effective in cases with a low tumor burden.

The autologous whole cell vaccine contains mainly tumor but also some normal tissues. Autoimmune disease is therefore a possible adverse effect of active immunotherapy. Melanoma vaccines have caused vitiligo after vaccination in some trials (30, 31). Antigen-presenting cells may present hepatitis virus antigens within tumor tissue and induce specific antiviral immunity, whereas the activated CTLs may consequently attack virus-infected hepatocytes and result in possible impairment of liver function. In our study, however, we did not observe any occurrence of autoimmune disease or exacerbation of hepatitis after vaccination. These findings, consistent with the results from our prior study (18), indicate that AFTV therapy is safe for clinical use. Since the beginning of the Phase I clinical trial 3 years ago, grade 1 or 2 skin toxicities have been the only adverse effects observed. Feedback from all patients indicates that quality of life is not negatively influenced by this treatment.

In addition to its safety, AFTV therapy is easily administered at bedside on an outpatient basis because hospitalization (suggested for many adjuvant therapies) is unnecessary. AFTV could be administered in many medical institutes where MHC-matched peptide vaccine preparation, complicated recombinant techniques, or live cell culture is impractical. In addition, AFTV is also applicable for treatment of other solid tumors that retain their antigenicity after formalin fixation.

Adjuvant immunotherapies for melanoma decreased recurrence rate after resection (32, 33) with some controversy on overall survival (34). However, to our knowledge, no adjuvant vaccination of autologous HCC has been published. The results of the present clinical trial, especially regarding overall survival (Fig. 3), will be an important milestone for further investigation of mechanisms of tumor vaccination, for example, on specific CTL level in various T lymphocytes.

In summary, AFTV therapy reduced early postoperative recurrence of HCC and improved overall survival. The best outcomes were observed in patients with low tumor burdens. These randomized Phase II trial results provide support for large-scale active immunotherapy treatment of HCC in hepatitis B-endemic countries.

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