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Research Article

The Effect of Adoptive Immunotherapy after Percutaneous Microwave Ablation in Recurrent Hepatocellular Carcinoma Patients with Hepatitis B: A Preliminary Study

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Abstract

To observe the influence of adoptive immunotherapy combination with percutaneous microwave ablation (PMWA) on peripheral blood examination, hepatic function examination and serum alpha fetoprotein (AFP) in recurrent hepatocellular carcinoma (HCC) patients with hepatitis B. Fourteen recurrent HCC patients with 31 lesions (D≤6.0cm, fewer than four tumors) were treated with radical PMWA and continuous four courses of adoptive immunotherapy, which were administrated at 1, 2, 3 and 4 months after PMWA, respectively. Under sonographic guidance, tumor lysate-pulsed DCs (1ml) were injected into bilateral groin lymph nodes at 9th day, while CTL (25ml) were injected into the abdominal cavity at 11th day and CIK (100ml) was infused intravenously at 14th day after hemospasis in one course of treatment, respectively. Peripheral blood examination, serum AFP and hepatic function were reviewed 1, 3, 6 months after adoptive immunotherapy. The number of white blood cell (WBC), lymphocyte (LYM), serum albumin (ALB) and cholinesterase (CHE) were detected increase significantly at 3 and 6 months after therapy compared to pre-therapy (p<0.05). Platelet (PLT) was detected increased significantly at 6 months after therapy (p<0.05) compared to pre-therapy. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were detected decreased significantly at 1, 3 and 6 months after therapy (p<0.05). As to serum AFP, it was detected decreased gradually, while there was no difference during the follow-up (6-16 months). No severe adverse effects were observed. Adoptive immunotherapy prescribed soon after PMWA was safe and ameliorated the laboratory examination and the immunity status of recurrent HCC patients, which may improve the prognosis.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most aggressive and prevalent malignant diseases worldwide and characterized by a dismal prognosis. The incidence and mortality of HCC are increasing yearly. Most of the burden (85%) is borne in developing countries, with the highest incidence rates reported in regions where infection with hepatitis B virus (HBV) is endemic: Southeast Asia and sub-Saharan Africa [1-3]. Although current therapeutic modalities including liver transplantation, surgical resection, radiofrequency ablation (RFA), microwave ablation (MWA), percutaneous ethanol injection (PEI) and transcatheter hepatic arterial chemoembolization (TACE) have significantly improved in the last few decades and shown survival benefits for HCC patients, the survival rate is still unsatisfactory [4]. The main reason is the high recurrence rate after treatment [5, 6]. And the HCC patients are notoriously resistant to chemotherapy and radiotherapy [7]. So far, efficient therapeutic strategy to recurrent HCC patients is needed to be solved urgently. Meanwhile, recurrent HCC patients demonstrate some disordered in their blood subsets and hepatic function examination. This makes the antitumor effect reduced greatly. And a poor physical condition of recurrent HCC patients cannot afford invasive treatment. And medication-assisted therapies itself increase the burden on liver and damage the hepatic function further. This may contributes to the high recurrence rate after therapy. So searching therapeutic strategy both to improve the antitumor effect of recurrent HCC patients and to ameliorate the laboratory examination is imperative.

Liver is an immunity organ itself [7], immunotherapy is an alternative promising treatment strategy for many cancer types including HCC. It aims to provide or enhance innate or adoptive immunity against malignancies by harnessing the immune system to target tumors [9]. Several lines of evidence indicated that immunotherapy was often a synergistic partner for efficacy in combinations therapy without additive toxicity [10, 11]. Advances in the immunotherapy of cancer have also been met with a number of setbacks and achieved preliminary clinical efficacy in primary HCC [8, 12]. The adoptive immunotherapy focuses on adoptive immune system (T and B cells), including cytotoxic T lymphocytes (CTL), cytokine-induced killer cells (CIK), dendritic cells (DCs), tumor-infiltrating T lymphocytes (TIL) and so on [13]. The activation of tumor-specific CTL requires three synergistic signals: the presentation of tumor antigen by antigen-presenting cells (APC) to specific T-helper cells, the interaction between costimulatory factors and the secretion of immunostimulatory cytokines from activated T-helper cells [14]. DCs are potent professional APCs that can capture, process and present antigens. They are critical for exerting T-cell-mediated immune responses, activating naïve T cells and playing a critical role in innate immune response and adoptive immune response [15]. CIK cells are shown to be a heterogeneous population of which majority expresses both the T-cell marker CD3 and natural killer cell marker CD56. CTL and CIK cells are effective antigen-specific and

-nonspecific tumor-killing effectors. Adoptive immunotherapy with mature autologous DCs pulsed with tumor lysate ex vivo or antigen-specific and -nonspecific antitumor effectors have been demonstrated with variable efficacy. Adoptive immunotherapy with DCs, CTL and CIK has been demonstrated with obvious clinical effect [16, 17].

Percutaneous microwave ablation (PMWA) as a thermal ablation method not only reduces tumor load, but also can remove immunosuppression from tumor by enhancing release and exposure of tumor-related antigens from the death and apoptosis tumor cells, which further might help to overcome the immune tolerance towards the tumor. PMWA has achieved a satisfactory clinical efficacy in primary and recurrent HCC patients [18, 19]. Therefore, such immune-stimulating therapeutic interventions in combination with immunotherapy strategy represent a promising future approach for HCC treatment [13, 20].

This preliminary study was a combination therapy of PMWA and adoptive immunotherapy for recurrent HCC patients, which applied multiple immunocytes infusion including mature DCs, CTL and CIK. The aim was to observe it`s safety and influence on peripheral blood routine, serum alpha-fetoprotein (AFP) and hepatic function examination in recurrent HCC patients with hepatitis B.

Material and method

Patients

From August 2011 to November 2012, 14 patients (10 male, 4 female) with recurrent HCC aged 40 - 80 years (mean 59.8 ± 11.9 years) were treated with PMWA by the guidance of sonography or contrast-enhanced sonography (CEUS) and continuous four courses of adoptive immunotherapy, which were administrated 1, 2, 3 and 4 months after PMWA. The study was approved by the local research ethics committee of Chinese PLA General Hospital. Written informed consent was obtained from all patients before their enrollment into the study. Inclusion criteria included the followings: 1) the diameter of single nodular ≤ 6cm and the number of nodular ≤ 4; 2) absence of portal vein thrombosis or extrahepatic metastases; 3) Child-Pugh classification A or B; 4) without clinically significant ischemic heart disease or cardiac failure; 5) tumor accessible via a percutaneous approach; 6) cirrhosis with chronic hepatitis B; 7) expected survival of more than six months; 8) be able to comply with the immunotherapy program strictly. Laboratory examinations necessary for inclusion were as follows: WBC ≥ 2 x 10^9/L; PLT ≥ 75 x 10^9/L; the proportion of PLM ≥ 20%. Exclusion criteria: 1) the expectation survival of the recurrent HCC patients is less than 3 months; 2) the patients who is not able to comply with treatment. The characteristics of 14 recurrent HCC patients recruited into the study were listed in Table 1. Before receiving combination therapy, 8 patients received liver surgical resection, 4 patients PMWA or PRFA and 2 patients TACE. Although all patients in the study were HBV related, the HBV viral burden examination was lower than 500 (copies/ml). For inducing the hepatotoxicity of antiviral drug, all patients were not received antiviral therapy for HBV.

Table 1 Clinical data of fourteen recurrent HCC a patients

Num	Gen	Age	HBV history (years)	Num of nodules	Max diameter (mm)	differentiation	First-treament	*Recurrent time (months)	Child-plug
1	M	46	20	1	15	well	Surgical resection and TACE	5	5
2	M	74	32	3	35	moderate	PWMA	38	5
3	M	60	24	1	38	moderate	Surgical resection and TACE	4	4
4	F	61	17	1	12	poor	PRFA	8	6
5	M	54	26	3	30	well	Surgical resection and TACE	13	5
6	M	52	21	2	20	moderate	Surgical resection	21	5
7	F	69	39	1	41	moderate	laparoscope RFA	22	6
8	F	71	27	3	29	moderate	TACE	3	7
9	F	47	19	4	28	moderate	Surgical resection	25	4
10	M	70	23	2	52	well	PWMA	11	5
11	M	51	22	1	58	moderate	Surgical resection	61	5
12	M	80	41	2	24	moderate	TACE	1	6
13	F	40	13	3	29	poor	PWMA	9	5
14	M	62	33	4	35	moderate	Surgical resection and TACE	12	5

*Recurrent time: the recurrent time after the first-treatment

Microwave ablation equipment and techniques

Two patients with 4 nodules, 4 patients with 3 nodules, 3 patients with 2 nodules and 5 patients with single nodule received PMWA successfully. All patients were recruited as inpatients. The treatments were performed at the authors' institution and were implemented by the guidance of sonographic or CEUS. General anesthesia was achieved by using a combination of two anesthetics (propofo and ketamine). Two types of microwave equipment, KY-2000 and KY-2100 microwave applicators (Kangyou Medical, Nanjing, China) with a frequency of 2450 MHz and 915 MHz separately, were used in the study [21, 22]. The applicators can deliver a maximum power of 100 W through one or two 15-gauge internally cooled antennae, and are equipped with a thermal monitoring system connected to three 21-gauge tissue thermal monitoring needles.

Ablation treatment was designed and performed to ablate tumors completely with a single treatment session. A detailed protocol was worked out for each patient based on the size and position of the tumor, characteristics of the thermal field distribution of microwave antenna, and the authors' clinical ablation experience of 20 years [23]. In general, for tumors < 1.7 cm in diameter, a single antenna was used; for tumors ≥1.7 cm, multiple antennae were applied synchronously or consecutively. An output setting of 60 W for 300 seconds was routinely used. With sonographic guidance, one to three 21-gauge tissue thermal monitoring needles were placed at different sites 0.5 cm outside the tumor to monitor temperature throughout the procedure. The treatment session was ended if the hyperechoic region observed by sonography covered the entire tumor, or if the monitored temperature reached 60 ℃, or remained at 54 ℃ for at least 3 minutes. Otherwise, an increase in application time was required depending on the temperature which was monitored dynamically [23, 24].

Immunotherapy protocol

Four continuous courses of adoptive immunotherapy were performed from 1 to 4 months after PMWA, and which course lasts 14 days listed in Fig. 1. Tumor tissue was obtained by sonography-guided biopsy using an 18-gauge needle before PMWA for pathologic diagnosis and lysate preparation. Before every course, patients were checked review imaging and blood examination without new lesion or recurrence, and then the hemospasia were carried out. Immunocytes were separated and differentiated from peripheral blood mononuclear cells (PBMCs) in 50 ml peripheral blood. Under sonographic guidance, mature DCs (mDC) cells ($1\text{-}2\times10^8$) which cultured mature on 9th days in 1 ml physiologic saline was injected into bilateral groin lymph nodes (0.5 ml for each), CTL (DC-CIK) cells (20ml, $1\text{-}5\times10^9$) were injected into right upper abdominal cavity after 11th days (Fig. 2) and CIK cells (100ml, $1\text{-}5\times10^9$) were infused intravenously after 14th days after haemospasia, respectively.

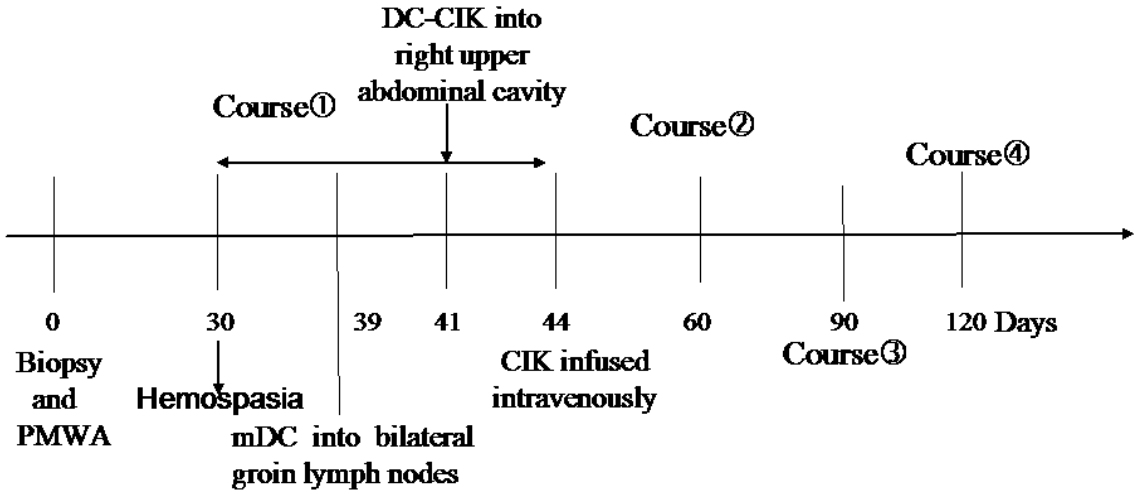

Figure 1 The combination therapy of PMWA and adoptive immunotherapy protocol. This figure shows that the flow chart of PWMA and continuous four courses adoptive immunotherapy subsequently.

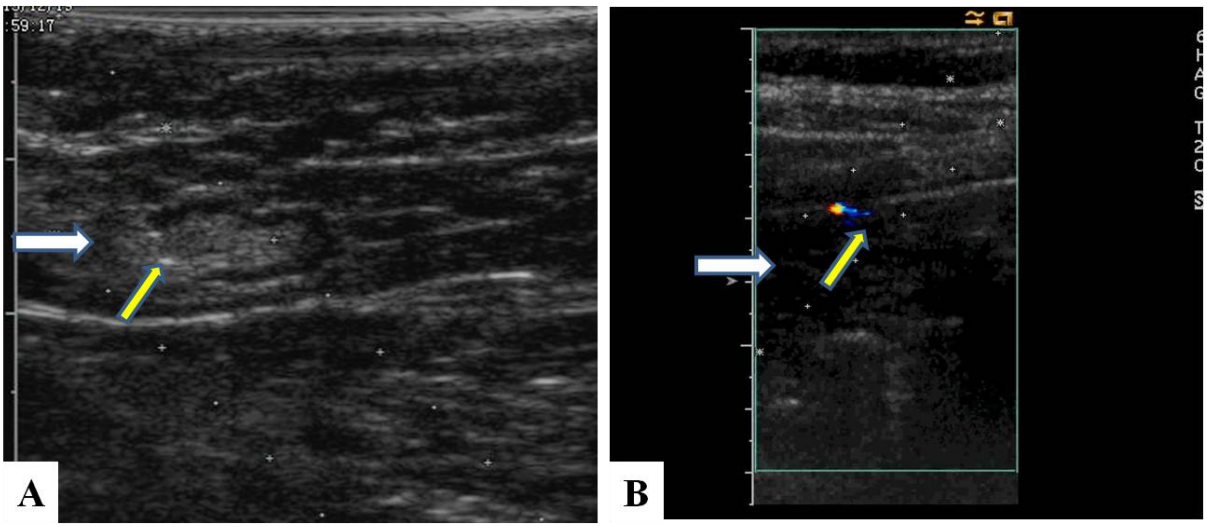

Figure 2 Immunocytes injected into the bilateral groin lymph nodes (A, white arrow: lymph nodes; yellow arrow: 21G PTC-B needle) and right upper abdominal cavity (B, white arrow: cavity; yellow arrow: cell persusion)

Preparation of DCs and effector cells from peripheral blood

Immunocytes were prepared according to the method of Romani in a Good Manufacturing Practice (GMP)-compliant facility [25]. PBMCs were collected via density gradient centrifugation over Ficol-Paque, and were suspended in 10ml STEM-34 and lay aside for 3 hours to adhere onto a plastic surface. After non-adherent cells were removed, the adherent cells were cultured at 37℃ in STEM-34 supplemented with 1,000U/mL granulocyte-monocyte colony-stimulating

factor and 500U/mL IL-4. Fifty percent of DC medium was changed every other day and the medium was supplemented which cytokines. Immature DCs were harvested on day 9 for injection and pulsed with antogenic tumor lysate before undergoing a maturation step in culture medium containing tumor necrosis factor α. To generate CIK cells, non-adherent cells were cultured at 37℃ in KBM-551 serum-free medium supplemented with 1,000U/mL interferon γ on the initial day. After 24 hours of incubation, 2ug/ml mAb CD3, 1ug/ml CD28 mAb, and 500u/mL IL-2 were added. Fresh IL-2 and fresh media were replenished every other day. CIK cells were harvested on day 14 for injection.

Pathological diagnosis, clinical observations and follow-up

Histological diagnosis was obtained by biopsy in all patients. Laboratory examinations, sonography, CEUS, contrast-enhanced CT and/or MRI were performed before and 1, 3, 6months and every 6 months subsequently after the combination therapy. Laboratory examinations included peripheral routine blood examination, serum AFP and hepatic function.

Statistical analysis

The statistical analysis was performed using SPSS version 16.0 (Chicago, IL, USA). Continuous data were expressed as means ± standard deviations (SD) and median. For comparison between multiple groups, analysis of variance or rank sum test were carried out as appropriate. For comparison between two groups, independent-samples t-test, rank sum test and Chi-square test were carried out. Two-tailed $p < 0.05$ was judged to be significant.

Results

Toxicity assessment

No serious adverse events occurred in a total of 168 times of cell infusions (three times of cell infusions in one course). A total of 26 adverse events were recorded. Adverse events included grade I/II fever defining as ≤ 38.5 ℃ (21 times, 32.8%) that could be recovered without deal with in 6-24h, and malaise (5 times, 7.8%) that could be recovered without deal with in 2–24 hours. There were no significant hepatic, renal, pulmonary, cardiac, hematological or neurological toxicities attributable to the cell infusions. No clinical manifestation of autoimmune reaction was observed.

Changes in routine blood examination

As shown in Table 2, the number of WBC, LYM and PLT of peripheral routine blood examination was detected increased significantly. As to WBC, there was significantly increased at 3 and 6 months post-therapy compared with pre-therapy examination ($p<0.05$). For LYM, compared with that of pre-therapy, 3 and 6 months post-therapy examination results showed obviously increased ($p<0.05$). Compared with pre-therapy PLT examination, 3 months post-therapy examination results showed a increased trend and it showed significantly statistical difference at 6 months ($p<0.001$).

Table 2 Phenotype of peripheral blood examination analysis before and 1, 3, 6 months after combination therapy

Phenotype / Time points	WBC (10^9/L)	LYM (10^9/L)	PLT (10^9/L)
Pre-therapy	4.23±0.74	1.37±0.53	118.79±44.47
Post-therapy			
1 month	4.56±1.18	1.60±0.49	124.50±50.31
3 months	5.06±1.22*	1.85±0.67*	129.79±54.16
6 months	5.18±1.22*	2.12±1.01*	166.64±51.73**

*Significantly difference was detected, $p < 0.05$

** Significantly difference was detected, $p < 0.001$

Changes in hepatic function examination

As shown in Table 3, compared with pre-therapy hepatic function examination, the post-therapy examination has improved greatly. For ALT, the examination of 1, 3, 6 months post-therapy have decreased sharply with a significantly difference (p<0.05) compared with that of pre-therapy. As to AST, the examination of 1, 3, 6 months post-therapy have decreased radically with a significant difference compared with that of pre-therapy (p<0.05). To ALP, it was detected an sharply decreasing trend till to 6 months post-therapy with significant difference (p<0.001) compared with pre-therapy. For serum albumin (ALB), the 3 and 6 months post-therapy examination have increased sharply with a significant difference compared with that of pre-therapy (p<0.001). To CHE, the examinations of 3 and 6 months post-therapy have increased sharply with a significant difference compared with that of pre-therapy (p<0.05). As to TBIL and DBIL, the 1, 3 and 6 months post-therapy examination results showed a decreased trend compared with that of pre-therapy, but with no significant difference.

Changes in alpha fetoprotein (AFP)

Compared with pre-therapy AFP examination (146.87 ± 229.16μg/L), the examination of 1, 3, 6 months post-therapy were 181.57 ± 329.47μg/L, 102.72 ± 167.94μg/L and 75.95 ± 142.07μg/L, respectively. Although there was a sharply decreased trend, the significant statistical difference was not detected (Table 3).

Table 3 Hepatic function analysis before and 1, 3, 6 months after combination therapy

Phenotype / Time points	ALT(U/L)	AST(U/L)	ALP(U/L)	ALB(g/L)	CHE(U/L)	TBiL(μmol/L)	DBiL(μmol/L)
Pre-therapy	56.25±60.24	62.77±88.17	89.51±29.96	38.99±4.17	5036±1914	17.41±9.69	6.62±4.08
Post-therapy							
1 month	45.14±49.90*	37.17±36.71*	96.77±27.82	39.61±5.68	5465±1685	18.25±8.57	6.60±3.67
3 months	30.42±16.70*	28.29±9.66*	84.39±23.37	43.10±5.16*	6125±1430*	16.15±8.08	6.26±3.36
6 months	18.80±14.02*	22.70±11.11*	55.31±20.29**	47.50±8.89*	6856±1669*	14.80±10.43	6.04±3.91

*Significantly difference was detected, $p < 0.05$

** Significantly difference was detected, $p < 0.001$

Discussion

As to recurrent HCC patients, the treatment was limited. Most patients have lost the opportunity of re-operation. While, PWMA as a safe, feasible and efficient minimally invasive method was widely used in clinical, and achieved gratifying results [18]. Recurrent HCC patients also face a higher risk of recurrence after re-treatment. How to prevent relapse in HCC has become the "bottleneck" of improving the prognosis. With the development of tumor immunology, a large number of basic and clinical researches confirmed that postoperative recurrence of HCC patients was correlated with their immune status. And adoptive immunotherapy could decrease postoperative recurrence and improve recurrence-free outcomes for HCC [26, 27]. For most of the recurrence of postoperative HCC occurs during the 6 months after operation. Adoptive immunotherapy can eliminate these residual cancer cells and destroy the proliferating cancer cells [12, 28]. Timely postoperative immunotherapy is necessary. So in this study, we investigate the feasible, safety and efficient of combination therapy with PWMA and adoptive immunotherapy in recurrent HCC patients.

The results of this study shown adoptive immunity could improve the number of WBC, LYM and PLT of peripheral routine blood examination greatly in recurrent HCC patients. It is reported that the number of WBC and LYM in peripheral were positive correlated to the immunity status [13]. As has reported, immunotherapy can improve the number and the phenotypes of lymphocyte which indicate the immunity status and the prognosis of cancer patients directly [29].

The number of PLT is related to hematopoietic system and splenic function in HCC patients. The majority of recurrence HCC patients were companied with esophageal gastric varices and hypersplenism, and lower PLT increases the risk of bleeding. Immunotherapy can increase the number of platelets and reduce the risk of bleeding, which benefits to the mortality of HCC patients.

PMWA can reduce the tumor burden and increase release of the tumor-associated antigen which further promotes the efficacy of immunotherapy. The synergy effect of immunotherapy and PWMA of liver tumor improve hepatic function significantly. This study showed ALT, AST, ALP, TBIL and DBIL have greatly decreased post-therapy. Among of the indexes, ALT, AST and ALP of have significant statistic difference compared to the examination of pre-therapy. Bilirubin is a major metabolite iron porphyrin compounds, which is toxic to brain and nervous system and can cause irreversible damage. Bilirubin is an important index for diagnosis of clinically jaundice and reflects hepatic function. And bilirubin is the main product of the spleen engulfing red blood cells, which reduced indicates the improvement splenic function and anemia. So the improvement in hepatic function may benefit to the life quality and prognosis of recurrent HCC patients. Because the serum CHE is synthesized by the liver, therefore decreased enzyme activity often reflects hepatic function damage. Meanwhile serum ALB is an indicator of liver protein synthesis function. So in this study, the serum CHE and ALB were increased obviously which reflected the promotion of hepatic function in recurrent HCC patients.

In this study, although the serum alpha-fetoprotein measurement of recurrence HCC patients post-therapy showed no significantly statistical difference comparing with pre-therapy, there was an obviously downward trend. Meanwhile, the relationship of serum alpha-fetoprotein measurement with the prognosis of HCC patients was reported yet [30].

Since HCC recurrence is just one of the causes of death in HCC patients, the other causes of death including hepatic function

failure, poor immunity status and infection, gastrointestinal bleeding and hepatic encephalopathy [31]. Therefore, by improving the patients' immune status and liver function can be achieved for improving the survival period of recurrent HCC patients. As a matter of fact, adoptive immunotherapy ameliorated some of the symptoms including increasing appetite, improving sleep, gaining body weight, and relieving pain [32]. Hence, immunotherapy may also improve the quality of life of postoperative patients.

However, this study was a preliminary one. The sample size was small, more significant changes may be seen by enlarging sample size. The follow-up period was rather short and the indexes that we used for evaluation need to be optimized. Longer follow-up period may show the benefits on the combination therapy of PWMA and adoptive immunotherapy to the prognosis of recurrent HCC patients.

In conclusion, this trial demonstrated that combination therapy of adoptive immunotherapy prescribed soon after PMWA for recurrence HCC patients was safe and ameliorated peripheral routine blood, hepatic function examination and serum alpha-fetoprotein measurement. Meanwhile these changes may improve the prognosis of recurrent HCC patients. The clinical effect should be further confirmed by a randomized controlled clinical trial with a larger sample size and a longer follow-up period.

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