Clinical Therapeutic Effect and Biological Monitoring of p53 Gene in Advanced Hepatocellular Carcinoma

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Objective: To investigate the therapeutic effect and biological changes of hepatic arterial infusion of p53 gene by the percutaneous port catheter system on advanced hepatocellular carcinoma (HCC) through a prospective randomized trial.

Methods: A total of 48 patients with advanced HCC between May 2005 and January 2009 were divided into the treatment group (30) and the control group (18). The port catheter system was implanted through the right external iliac artery approach in all the cases; the target artery was determined according to the manifestation of the angiograph. The patients in the treatment group were given arterial infusion of p53 gene (Gendicine, Shenzhen Sibiono GeneTech Co, Ltd) with Gendicine (10^{12}vp) combined with hydroxycamptothecin (20 mg), once a week, for a course continuously for 3 weeks. The arterial infusion with hydroxycamptothecin (20 mg) was given to the patients in the control group. Pretreatment/posttreatment a fetus protein and Karnofsky Performance Status values, change of tumor according to Response Evaluation Criteria in Solid Tumors (RECIST), and the survival time were analyzed. Pretreatment/posttreatment expression of mutant p53 gene and spontaneous micronucleus formation in the peripheral blood were evaluated by flow cytometry and micronucleus test in vivo.

Results: The patients in the treatment group received 1 to 8 courses of treatment, in which the differences between pretreatment/posttreatment AFP and KPS values were significant ($P < 0.05$), whereas there was no significant difference ($P > 0.05$) between pretreatment/posttreatment AFP and KPS values within the control group. After 1 month, the survival rates of the treatment and control groups (96.6% and 94.4%, respectively) and changes in the tumor evaluated according to RECIST were significantly different ($P < 0.05$) between the 2 groups. After 3 months, the survival rates of the treatment and control groups (83.3% and 55.6%, respectively) and changes in the tumor were also significantly different between the 2 groups ($P < 0.05$). After 6 months, the survival rates (50% and 11%, respectively) and changes in the tumor were significantly different between the 2 groups ($P < 0.05$). After 9 months, the survival rates (23.3% and 0%, respectively) and changes in the tumor were significantly different between the 2 groups ($P < 0.05$). Finally, after 12 months, the survival rates (6.67% and 0%, respectively) and changes in the tumor were significantly different between the 2 groups ($P < 0.05$). The difference between the pretreatment and posttreatment mean rates of p53 expression in patients in the treatment group was very significant ($P < 0.01$). The difference between the posttreatment mean rates of the treatment group and the control group was also significant.

Conclusions: Sequential therapy of p53 gene transcatheter arterial infusion was safe and could prolong the survival time of the patients. The biological study will play a positive role in guiding and monitoring the aspects of dosage selection and judgment of therapeutic efficacy.

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53 gene is one of the major tumor suppressor genes, and more attention has been paid on its inhibitory effect on hepatoma cells.[1,2] Today, interventional therapy is still the preferred and the most effective choice for patients with advanced and inoperable hepatocellular carcinoma (HCC). Hydroxycamptothecin, as a mild and effective alkaloid, is often used as well.[3] Interventional therapy with p53 gene in HCC, in contrast, has recently become the new focus of interventional therapy in HCC.[4,5] This study randomly selected a group of patients with advanced HCC and gave them sequential therapy of p53 gene transcatheter arterial infusion to investigate the clinical effect of the treatment and perform biological monitoring and evaluation of the effectiveness data in terms of cytology and molecular biology before and post interventional therapy.

MATERIALS AND METHODS

Clinical Data

The inclusion criteria included male and female patients aged between 18 and 75 years with confirmed diagnosis of primary HCC according to the Clinical Diagnosis and Staging Criteria of Primary HCC developed by the Chinese Society of Liver Cancer[6]; diffuse type or > 10 cm tumor diameter; hepatic function classified as Child-Pugh B or more severe and level of alanine aminotransferase of more than 4 times the normal range; Barcelona Clinical Liver Cancer stage of HCC of B or more; Eastern Cooperative Oncology Group perfor- mance status of 0 to 2; white blood cell count of > 4.0 10^9/L, hemoglobin level of > 100 g/L, and platelet count of > 80 10^9/ L; and significantly increased AFP (> 400 ng/mL). All patients in this study signed the same informed consent.

Background Data of Patients

Thirty patients (25 male and 5 female) with a mean age of 53 years (range, 34-76 y) having primary HCC during the period from June 2007 to May 2009 were randomly placed into the treatment group. In this group, 4 patients had postoperative multiple intrahepatic metastases and 3 patients had extra- hepatic metastases. Twenty-five patients could not receive curative treatment at the time of treatment. Twenty-six patients had stage C disease and 4 patients had stage B disease according to BCLC stage criteria (Table 1).

The control group consisted of 18 patients (14 male and 4 female) with a mean age of 51 years (range, 28-68 y) having primary HCC. In this group, 3 patients had postoperative multiple intrahepatic metastases and 1 patient had extrahepatic

TABLE 1. Background Data of Patients in Treatment Group

	n (%)
ECOG score, n (%)	
0	2 (6.6)
1	24 (80.0)
2	6 (20.0)
Child-Pugh score, n (%)	
A	22 (73.3)
B	8 (26.6)
BCLC stage, n (%)	
Stage B	4 (13.3)
Stage C	26 (86.6)
Earlier treatment, n (%)	
Surgery	5 (16.6)
Untreated	25 (83.3)
Metastasis, n (%)	
Intrahepatic metastasis	4 (13.3)
Extrahepatic metastasis	3 (10.0)

metastases. Fourteen patients could not receive curative treat- ment at the time of treatment. Sixteen patients had stage C disease and 2 patients had stage B disease according to BCLC stage criteria (Table 2).

The change of lesion was evaluated according to the RECIST (Response Evaluation Criteria in Solid Tumors) criteria.[7] There was no significant difference between the data of the 2 groups ($P > 0.05$).

Methods

Random allocation coding was used in the study, which were generated by computer simulation with SAS software by statisticians. A randomly assigned number was given to each enrolled patient, who was given treatment in the order of the time of visit by investigators.

Enhanced abdominal computed tomographic scan was taken before each interventional therapy. Digital subtraction angiography examination and interventional therapy were given according to the standard rule on interventional treat- ment for HCC (draft).[8] A Siemens Polystar-I digital subtrac- tion angiography machine was used to conduct conventional angiographies on the celiac artery, superior mesenteric artery, and indirect portal vein; the main feeding artery was selected as the target artery for drug infusion according to the findings on the angiograph. The distal end of the right external iliac

TABLE 2. Background Data of Patients in Control Group

	n (%)
ECOG score, n (%)	
0	1 (5.56)
1	20 (66.7)
2	5 (27.7)
Child-Pugh score, n (%)	
A	12 (66.6)
B	6 (33.3)
BCLC stage, n (%)	
Stage B	1 (5.56)
Stage C	17 (94.4)
Earlier treatment, n (%)	
Surgery	4 (22.2)
Untreated	14 (77.8)
Metastasis, n (%)	
Intrahepatic metastasis	3 (16.6)
Extrahepatic metastasis	1 (5.56)

artery was used in the study, a subcutaneous medicine box (Implantofix, B, Braun, German) was implanted above the right inguen, the indwelling catheter tip was left in the target artery, and the sequential infusion of medicine was given.

The p53 gene used in the study was recombinant adenovirus p53 for injection. The patients in the treatment group were given Gendicine (10^{12}vp) combined with hydro- xycamptothecin (20 mg), once a week, for a course continu- ously for 3 weeks. The duration of interval between the courses depended on the general physical condition, results of blood routine examination,

and liver and kidney functions of the patients; however, none of the intervals was less than 1 month. For the patients in the control group, the same administration route and pattern were followed. The therapeutic regimen was hydroxycamptothecin (20 mg), once a week, with a course continuously for 3 weeks.

Measurement of Mutant p53 Expression

We collected 5 mL of anticoagulated peripheral blood by an aseptic operation before the first intervention and after 4 interventions in each patient, which was then divided into 2 parts for end-point detection of different biological measure- ments.

The detection was completed with direct staining by a Flow Cytometer (Facs Calibur, BD [Becton, Dickinson and Company]). Mononuclear cells (or monocytes) was isolated with a lymphocyte separating solution using 1 mL of anti- coagulated peripheral blood, and was fixed in 70% alcohol overnight after a phosphate buffered saline (PBS) wash. The alcohol was removed by centrifugation; 90% of the leukocytes were removed by gradient centrifugation using the lymphocyte separating solution after PBS wash. Therefore, the remaining cells were monocytes, including tumor cells that possibly existed in the peripheral blood, and then the cell concentration was adjusted to 1 10^6/mL. The treatment solution was added with mouse-human mutant p53-fluorescein isothiocyanate monoclonal antibody by setting up a baseline control tube with a isotype agent, followed by a 30-minute reaction in dark and then analyzed after a PBS wash. The samples containing 10^4 cells/mL were automatically taken up by the Cellguest software and analyzed. The percentage of p53 positive cells was recorded.

Micronucleus Test of Human Lymphocytes

The slide for micronucleus test was immediately prepared after the collection of fresh anticoagulated peripheral blood. We added 0.1 mL of anticoagulated whole blood taken with 200 μL sample injector into a 0.5 mL tip. Then, we added 0.05 mL of 3% gelatin into the tube followed by a water bath at 37°C for 45 minutes, and subsequently aspirated the super- natant into another 0.5 mL tip tube for centrifugation, and then mixed the microprecipitate well using a shaker. Subsequently, we aspirated the solution with 10 μL of sample injector to prepare the blood slide, which was then fixed with 100% methanol and stained with Giemsa after air drying. The number of micronucleated cells in 2000 intact lymphocytes was recorded for each patient and was represented as percentage. Detailed information of analysis index and notes could be found in the reference section.[9]

Observation Indexes

General condition, AFP values, Karnofsky performance status (scores of patients who have or have not completed a course of treatment were evaluated at the end of the treatment), change of lesion (enhanced computed tomography was

TABLE 3. KPS, Survival Time, and AFP

	KPS		Survival Time	AFP	
Group (n)	Pretreatment	Posttreatment	Mean Survival Time	Pretreatment	Posttreatment
Treatment group (30)	58.8 ±14.8	66.8 ±8.4	199.6 ±111.8	45064.1 ±42349.8	33994.7 ±34750.8
Control group (18)	59.2 ±14.4	58.3 ±13.9	83.0 ±50.2	47573.9 ±39734.8	49042.5 ±37285.4

repeated on 1, 3, 6, 9, and 12 mo after surgery to investigate the change of tumor size; tumor size was calculated according to RECIST criteria, for example, the area of single tumor was the longest diameter multiplied by the maximum width of the vertical, and the area of multiple tumors was the total of each tumor area), and survival time before and after treatment were evaluated in all the patients, and the rate of mutant p53 expression and spontaneous micronucleus formation (MNF) in vivo before and after treatment were evaluated in patients in the treatment group.

Statistical Analysis

Statistical analysis was carried out with SPSS 13.0 software; 2 independent sample tests of the nonparametric test was used in comparison with survival time, Karnofsky performance status, and AFP between the 2 groups, and survival curve was calculated on the basis of Kaplan-Meier method. Ranking test was used in comparison with the therapeutic effect. Two related sample tests of the nonpara- metric test were used in comparison with AFP measured before and after treatment in each group of patients for which statistical significance was defined as $P < 0.05$. For patients in the treatment group, the difference between the rates of mutant p53 expression before and after the treatment was evaluated with t test (statistical significance was defined as $P < 0.05$).

RESULTS

Clinical Observation

Thirty patients with primary HCC were randomized into the treatment group, 22 of them were male, 8 of them were female, with age ranging from 35 to 76 years. Nineteen of the

All patients in the treatment group achieved the treatment requirements and were not lost in follow-up.

Fever in varying degrees occurred after treatment in all patients in the treatment group, most of them had moderate- to-high-degree fever, but all were controlled. Eczema at the angles of the mouth occurred in 2 patients. Nausea, vomiting, diarrhea, or myelosuppression was not observed in any of the patients in both the groups. The median survival time of patients in the treatment group was 186 days, and that of the control group was 70 days. In 14 patients with moderate or severe ascites in the treatment group, ascites were significantly absorbed in 9 patients with the manifestations of no or mild ascites, whereas no such improvement was observed in 9 patients with moderate or severe ascites in the control group. No improved results of ALT were observed in either group. The results of Karnofsky performance status, changes of lesions (RECIST criteria), survival time, and changes in AFP values pre/post treatment in patients in the treatment and control groups are shown in Tables 3 and 4 and Figures 1 and 2.

Dynamic Change of Pre/Posttreatment Mutant p53 Expression With p53

Eighteen patients in the treatment group received 4 or more interventions. The changes between pre/posttreatment p53 expression rates of the 18 patients are shown in Table 5. The mean rate of mutant p53 expression after intervention of p53 gene transcatheter arterial infusion significantly decreased, which resulted in a statistical difference when compared with that before treatment with therapies (pre/posttreatment mean expression rates were 23.74% and 11.81%, respectively) ($P < 0.01$).

30 patients in the treatment group had massive-type disease with a diameter of 10 to 16 cm and a tumor thrombus in the main portal vein and in the left and right branches of the portal vein; 10 of them had hepatic arterioportal fistulas and 11 had diffuse-type disease. Fourteen patients had moderate or severe ascites. The hepatic function was classed as Child-Pugh B in 18 patients and Child-Pugh C in 12 patients. Alanine aminotransferase (ALT) increased by 4 fold of the normal value in 26 patients. AFP level was significantly increased in 28 patients (> 400 ng/mL). Eighteen patients had IIIa stage disease, whereas 12 patients had IIIb stage disease.

The control group had 18 patients with primary HCC (14 male and 4 female) with age ranging from 28 to 68 years. Eleven patients in the control group had massive-type disease with a diameter of 10 to 16.5 cm and a tumor thrombus in the portal vein; 7 of them had hepatic arterioportal fistulas and 7 had diffuse-type disease. Ten patients had ascites. The hepatic function was classed as Child-Pugh B in 11 patients and Child-Pugh C in 7 patients. ALT increased by 4 fold of the normal value in 17 patients. AFP level was significantly increased in 17 patients (> 400 ng/mL). Eleven patients had IIIa stage disease, whereas 7 patients had IIIb stage disease.

TABLE 4. Comparison of Lesion Changes of 2 Groups at Different Time Points (1, 3, 6, 9, 12 mo After Treatment), all $P < 0.05$

Treatment Group	1 mo	3 mo	6 mo	9 mo	12 mo
Patients alive (n)	29	25	15	7	2
Survival rate	96.60%	83.30%	50%	23.30%	6.67%
CR	0	0	0	0	0
PR	4	4	3	0	0
NC	21	11	4	2	2
PD	4	10	8	5	0
Control group					
Patients alive (n)	17	10	2	0	0
Survival rate	94.40%	55.60%	11.10%	0.00%	0.00%
CR	0	0	0	0	0
PR	0	0	0	0	0
NC	10	2	0	0	0
PD	7	8	2	0	0

CR indicates complete response; NC, no change; PD, progressive disease; PR, partial response.

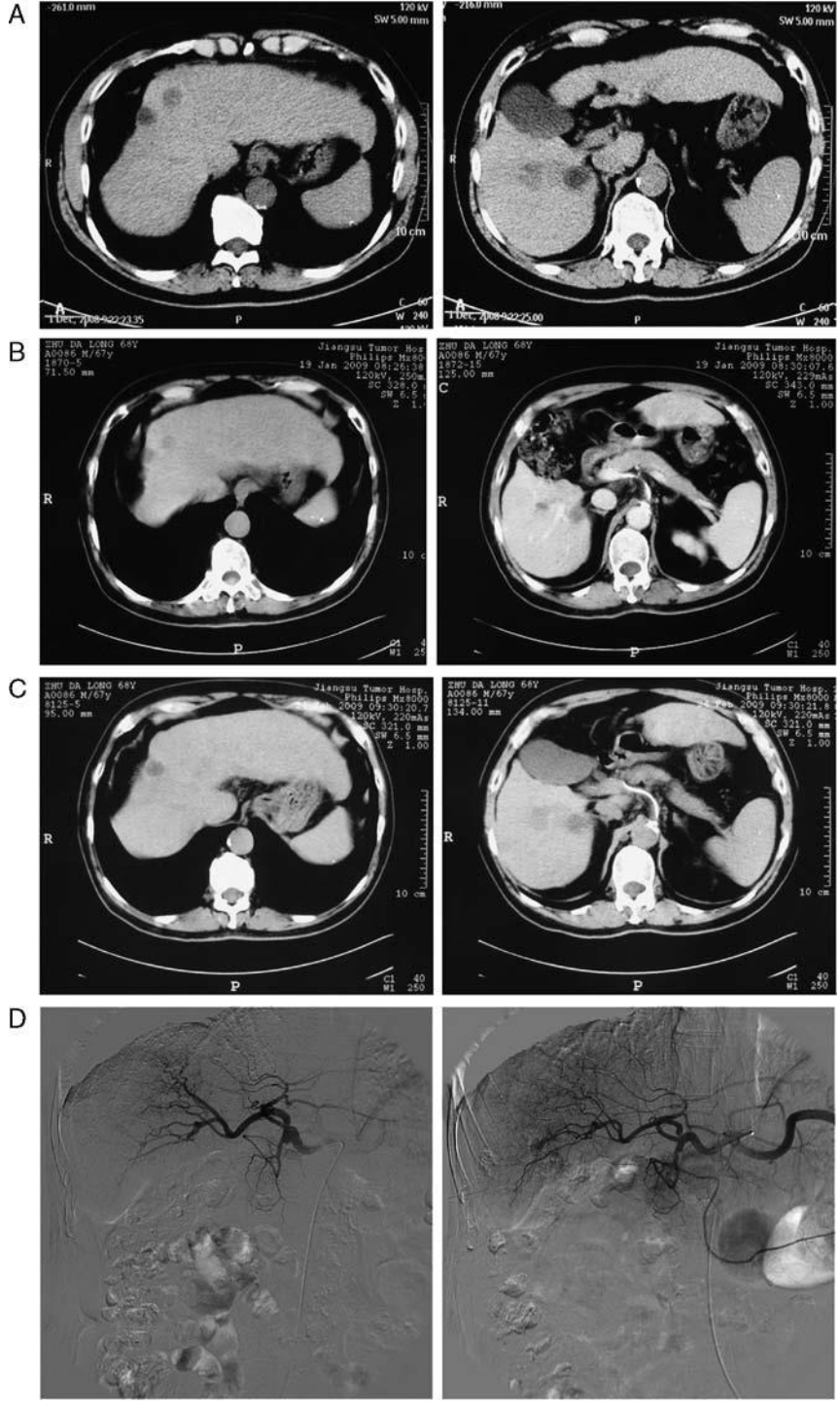


FIGURE 1. A to D, shows the result of 1 patient. A, A 68-year-old man was admitted to the hospital owing to a focal liver lesion for 14 days; AFP: > 363 ng/mL; diagnosis of CT: the lesion was considered as nodular HCC. The larger lesion, 3.2 3.3 cm, located in the area of light hepatic lobe was near to the diaphragmatic dome. B, imaging of CT reexamination after 49 days. C, imaging of CT reexamination after 92 days. D, there were no significant progress of tumor size and no visible lesion in the liver according to the 2 photographs of DSA taken after the first implantation of the medicine box and second infusion therapy days. Note that there are 3 issues with CT imaging: (1) the selected lesion slice and time phase were not consistent with each other; (2) the time phases of enhancement scanning were not uniform; (3) the size of images in Figure 1A was different from that in Figures 1B and C. We considered no change of lesion based on earlier experiences. The tumor was not considered to be hypervascular as no manifestation of tumor staining of the lesion was observed; thus, we could judge the change of tumor lesion size according to the findings of DSA. The patient probably had cholangiocellular carcinoma. CT indicates computed tomography; HCC, hepatocellular carcinoma.

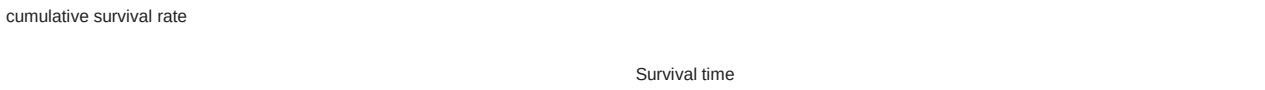


FIGURE 2. Kaplan-Meier survival curves of patients in 2 groups.

Change Between Pretreatment/Posttreatment Values in Spontaneous MNF

The mean spontaneous MNF before intervention with p53 and the mean MNF after 2 to 4 interventions in 48 patients with advanced HCC were both significantly increased in the treatment group compared with the control group (both $P < 0.01$); the mean pretreatment/posttreatment MNFs also had significant statistical difference ($P < 0.05$), as shown in Table 6.

There was no significant difference between the pretreat- ment Karnofsky performance statuses of the treatment and the control groups ($P > 0.05$), but the difference between the posttreatment Karnofsky performance statuses between the 2 groups was significantly different ($P < 0.05$). The survival time comparison was as follows: $P < 0.05$; comparison of pretreat- ment AFP of both the groups: $P > 0.05$; and comparison of pretreatment AFP of both the groups: $P < 0.05$.

DISCUSSION

Transcatheter arterial chemoembolization (TACE) is an important type of HCC treatment, with definite therapeutic effect especially in patients with moderate-to-advanced, inoperable HCC. There were, however, contraindications or relative contraindications of TACE in HCC, such as severe hepatic and renal dysfunction, severe jaundice, severe ascites, complete occlusion of the main portal vein, arterio-venous fistula, tumor whose volume was more than 70% of total liver volume, and patients with very poor general condition. For these patients, only a conservative, symptomatic supportive treatment could be given, by which few of the patients could improve and regain the chance of performing TACE, and most of them had disease progression and a very short survival time with a mean time of 1 to 4 months. No ideal clinical treatments are currently available for such patients.

The development of HCC is a complicated, multifactor, multigene, multistage, and multipathway process, and is one of the results of the effects of nature and nurture risk factor interactions on the stability of the genome in somatic cells. p53 gene is an important tumor suppressor gene located on human

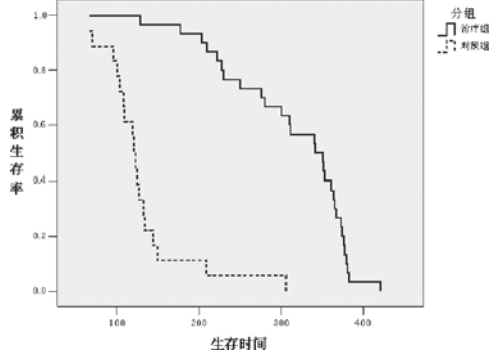
TABLE 5. Dynamic Change Between Preintervention/ Postintervention p53 Expressions of Patients

TABLE 6. Comparison of Spontaneous Micronucleus Formation Between Patients With Hepatocellular Carcinoma and Natural Population in Control Group

Group (n)	Rate of MNF (%)	*P* (Compared With Pretreatment)
Healthy adult (48)	0.47 ±0.88	---
Preintervention (28)	2.05 ±1.67	< 0.01
Preintervention (20)	1.44 ±1.12	< 0.01, < 0.05*

*Compared with pretreatment result. MNF indicates micronucleus formation.

chromosome 17p13.1 with a total length of 16 to 20 kb, which contains 11 exons and 10 introns and codes 393 amino acids. Normal p53 gene, also known as the wild-type p53 gene (wt p53), can maintain the stabilization of genome, inhibit or prohibit cell transformation, and thus inhibit the development of tumor.[10] Change of p53 and loss of functional p53 protein can occur in at least 50% of malignant human tumors including HCC. Some HCCs contain a mutant allele and a normal allele, whereas other HCCs have a mutant and a missing allele. Thus, suppressor gene therapy is an essential approach to conquer cancer fundamentally, as normal tumor suppressor gene could substitute or compensate for the defective genes after introduction into tumor cells and subsequently inhibit tumor growth.[11] The study by Qiang et al[12] indicated the expression of recombinant adenovirus in the human body after trans- catheter arterial infusion targeted some organs, which occurred very early and maintained for a long time in the liver. The experimental study by Guang-Yu et al[13] confirmed the efficacy of transcatheter arterial infusion therapy with p53 gene. The study by Yong-Song et al[14] documented the close association between p53 antibody and clinical features of HCC. Many existing studies are still basic animal experiments and in vitro studies, and there are rare applications in clinical experiments. Our study provided an initial report on the clinical application of p53 gene. All the patients with HCC enrolled in this study had no Child-Pugh A hepatic function and large or diffuse lesion accompanied with a tumor thrombus in the portal vein and arterio-venous fistula. All the patients were inoperable, and could not obtain ideal efficacy but had high risk from conventional TACE. We used recombinant adeno- virus as vector to perform p53-targeted therapy by sequential transcatheter arterial infusion by the subcutaneous port catheter system implanted by the right external iliac artery approach to continuously transfer recombinant adenovirus-p53 injection in a short duration, thus considerably increasing the transfection efficiency of p53 gene in the target organ and subsequently reducing the number of interventional catheterization, making the therapy easily acceptable by the patients with advanced HCC. The study results show a prolonged survival time of the patients, decrease in AFP level, significant improvement of ascites in some patients, and no emergence of severe complications. The change of Karnofsky performance status indicated the improvement and increase in quality of life of the patients. According to RECIST criteria, the size of lesion significantly decreased over a period of time, or there was less metastasis of lesion. Simultaneously, the expression of mutant p53 gene in the peripheral blood of treated patients considerably decreased with a very significant statistical difference ($P < 0.01$) compared with the pretreatment measure- ment result. In addition, the rate of spontaneous MNF also showed a decreased tendency compared with pretreatment



Time (n)	p53 Expression Rate (%)	*P*
Preintervention (18)	23.74	---
Postintervention (18)	11.81	< 0.01

result with a significant difference ($P < 0.05$); the dynamic change of p53 expression in the peripheral blood and MNF were closely associated with the relief of clinical symptoms in patients. There were no reports similar to this result in existing similar studies. Mutated p53 protein of free tumor cells in the peripheral blood had a longer half-life and disappeared (though not easily) from the circulating peripheral blood. However, some tumor cells presented in peripheral blood circulation might be in apoptotic phase. In such cells, the components have changed, p53 protein still existed which could have positive results by sensitive detection. Furthermore, the presence of spontaneous MNF in the human body, which served as biological markers of chromosome damage, genomic instability, and risk factors of end-stage tumor, was the comprehensive reflection of chromosome instability phenotype and change of cell survival resulting from gene deletion and exogenous mutagens.[15]

In conclusion, p53 gene therapy is a positive and effective treatment for patients with advanced HCC with poor general condition. The further evaluation of dosage and efficacy of gene therapy in advanced HCC treatment based on experimental data obtained from biology monitoring will be conducted to provide positive biological guidance and monitoring role.

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