

## Original Research Article

# Effect of Yugan Sanjie decoction on expressions of regulatory T cells, serum P21 protein and vascular endothelial growth factor in mice with hepatocellular carcinoma

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### Abstract

**Purpose:** To investigate the effect of Yugan Sanjie decoction on the expressions of regulatory T cells (Tregs), serum P21 protein and vascular endothelial growth factor (VEGF) in mice with hepatocellular carcinoma.

**Methods:** A total of forty specific-pathogen-free (SPF) Kunming mice were randomly assigned to four groups of 10 mice each. Except for normal control group, the other three groups were transfected with hepatoma-22 (H22) cells to establish a mouse model of liver cancer. Mice in the cyclophosphamide group was given cyclophosphamide at a dose of 20 mg/kg daily intragastrically, while those in decoction group were treated with Yugan Sanjie decoction (0.4 ml/kg/day) intraperitoneally. After 30 days of treatment, serum levels of CD4<sup>+</sup>Th17, CD4<sup>+</sup>CD25<sup>+</sup> Treg, Th17/Treg, TNF- $\alpha$ , and VEGF were determined.

**Results:** There was lower serum level of CD4<sup>+</sup>Th17 in the decoction group than in negative control and cyclophosphamide groups ( $p < 0.05$ ). However, higher serum levels of CD4<sup>+</sup>CD25<sup>+</sup> Treg and Th17/Treg were seen in the decoction group, relative to the negative control and cyclophosphamide groups ( $p < 0.05$ ). Serum TNF- $\alpha$  was also markedly elevated in decoction group, when compared with negative control and cyclophosphamide groups ( $p < 0.05$ ). Serum VEGF was markedly lower in decoction group than in negative control and cyclophosphamide groups, and was appreciably lower in cyclophosphamide group than in negative control group ( $p < 0.05$ ).

**Conclusion:** Yugan Sanjie decoction effectively alleviates clinical symptoms of LC, and improves immune function of mice by regulating serum levels of T lymphocytes. These findings provide scientific support for a new treatment strategy.

**Keywords:** Liver cancer, Yugan Sanjie decoction, Regulatory T cells, P21 protein, Vascular endothelial growth factor

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## INTRODUCTION

Liver cancer (LC) is a malignant tumor characterized by slow onset and rapid

progression. It is ranked second in the hierarchy of malignant tumors. The early symptoms of LC are generally not obvious until the disease reaches an advance stage. The moment the

symptoms become obvious, the tumor is already large, and the patient will usually suffer from cachexia and die within a few months. Surgery and conservative treatment are two major strategies for treating LC [1,2].

In Traditional Chinese Medicine (TCM), LC is described as "liver accumulation, jaundice, abdominal mass, and liver fixity". It is believed that the onset of the disease is related to lack of vitality and dysfunction in the body, and the treatment involves the use of drugs that strengthen the spleen, promote blood circulation, remove phlegm, resolve phlegm and stasis, and soothe the liver [3]. Studies have shown that treatment strategies adopted in TCM for treating LC improve patients' quality of life, prolong survival, and control disease progression.

*Yugan Sanjie* decoction consists of thirty different types of Chinese herbs. It functions in detoxification, clearing away *heat*, soothing liver and dampness, and it can be used along with radio- or chemotherapy. It is clinically effective with little or no side effects [4]. The aim of the present study was to investigate the effect of *Yugan Sanjie* decoction on the expressions of Tregs, serum P21 protein and VEGF in mice with hepatocellular carcinoma.

## EXPERIMENTAL

### Experimental mice

A total of forty specific-pathogen-free (SPF) Kunming mice weighing 15.9 to 22.7 g (mean weight =  $20.5 \pm 1.6$  g) and aged 3 to 4 weeks, were used in this study. They were housed in metal cages under standard conditions of animal care and provided standard feed and water *ad libitum*. The mice were randomly assigned to four groups (10 mice per group): normal control group, negative control group, cyclophosphamide group and decoction group.

This research received approval from the Animal Ethical Committee of Department of Oncology, Yantai Hospital of Traditional Chinese Medicine (approval no. 201511936), and was conducted according to the guidelines of "Principles of Laboratory Animal Care" (revised NIH publication 85-23 [5]).

### Establishment of mouse model of LC

Except for the normal control group, all groups were transfected with hepatoma-22 (H22) cells to establish mouse model of LC. Cells in exponential growth phase were collected for injection via trypsinization. Cell suspension in a

volume of 100  $\mu$ l was adjusted to the required density ( $1 \times 10^6$  cells) for inoculation, and was injected subcutaneously into the hind leg of each mouse, to establish an athymic nude mice with survival of 10 - 12 weeks. The injection sites were monitored until tumors were established. Digital calipers were then used to measure the tumors until they reached sizes of 100 - 200  $\text{mm}^3$ .

### Preparation of *Yugan sanjie* decoction

The decoction consisted of *Isatidis Radix* (30 g), *Astragalus* (30 g), *Atractylodes macrocephala* (15 g), *Smilax glabra roxb* (15 g), *Curcuma aromatica* (15 g), *Paeonia albiflora pall* (10 g), *Aurantii fructus* (12 g), *Polygonati rhizoma* (30 g), *Bupleurum* (10 g), *Salvia miltiorrhiza* (20 g), *Jiaoshanzha* (15 g), and tangerine (10 g), all of which were dissolved in 1000 ml of distilled water.

### Treatment regimen

The cyclophosphamide group received cyclophosphamide (20 mg/kg bwt/day) intragastrically. Mice in decoction group were treated with decoction of *Yugan Sanjie* (0.4 ml/kg bwt/day) intraperitoneally, while the normal and negative control groups did not receive any form of treatment. The treatment lasted for 30 days.

### Determination of tumor inhibition

Tumors removed from the mice were weighed using an electronic balance. The measurements were performed in triplicate, and the mean values were taken. Tumor inhibition (T) was calculated as shown in equation 1:

$$T (\%) = \{(1 - (T_e / T_c))\} 100 \dots\dots\dots (1)$$

where  $T_e$  = tumor weight in experimental group; and  $T_c$  = tumor weight in normal control group.

### Weight and number of spontaneous activities

After removal of tumors from the mice, they were weighed every morning using an electronic balance. A stopwatch was used to record the number of spontaneous activities of the mice within 5 min.

### Determination of serum levels of Tregs

Fasting venous blood (5 mL) was drawn from each mouse in the morning of sacrifice and centrifuged at 3000 rpm for 10 min to obtain serum which was used for assay of  $\text{CD}_4^+$  Th17,

CD4<sup>+</sup>CD25<sup>+</sup> Treg, Th17/Treg using a flow cytometer.

**Assessment of immune function**

Serum TNF-α and IL-2 were assayed with appropriate ELISA kits.

**Determination of serum levels of P21 protein and VEGF**

Serum P21 protein and VEGF were determined with ELISA kits.

**Evaluation of thymus and spleen functions**

Following 30 days of treatment, mice sacrifice was effected via cervical dislocation. The thymus and spleen were removed, washed with ice-cold physiological saline, and weighed using an electronic balance. The thymus (T) and spleen (S) function indices were estimated as shown in Eqs 2 and 3, respectively:

$$T = \{wt (mg)/bwt (g)\} \times 10 \dots\dots\dots (2)$$

$$S = \{ws (mg)/bwt (g)\} \times 10 \dots\dots\dots(3)$$

where wt = weight of thymus; bwt = body weight; ws = weight of spleen

**Statistical analysis**

Data are presented as mean ± SEM. Statistical analysis was carried out with SPSS version 19.0. Groups were compared using Student's *t*-test and χ<sup>2</sup> test. Statistical significance was fixed at *p* < 0.05.

**Table 1:** Profile of mice in the four groups (n, %)

Group	Tumor inhibition (%)	Survival (%)	Body weight (g)	Number of spontaneous activities
Normal control	-	100.00	25.36 ± 2.60	18.36 ± 2.95
Negative control	0.00 (0.00)	4.00 (40.00)	16.36 ± 2.98	2.50 ± 1.79
Cyclophosphamide	3.23 (32.26) <sup>*</sup>	6.00 (60.00) <sup>*a</sup>	20.38 ± 1.85 <sup>a</sup>	6.94 ± 1.23 <sup>a</sup>
Decoction	6.22 (62.15) <sup>*#</sup>	8.00 (80.00) <sup>*#</sup>	24.36 ± 2.70 <sup>#</sup>	12.36 ± 3.56 <sup>*#a</sup>

<sup>\*</sup>*P* < 0.05, when compared to negative control group; <sup>#</sup>*p* < 0.05, relative to cyclophosphamide group; <sup>a</sup>*p* < 0.05, relative to normal control group; n = 10

**Table 2:** Serum levels of Tregs (n, %)

Group	CD4 <sup>+</sup> Th17 (%)	CD4 <sup>+</sup> CD25 <sup>+</sup> Treg (%)	Th17/Treg
Normal control	0.97 ± 0.23	11.86 ± 0.95	33.26 ± 3.24
Negative control	2.42 ± 0.83	4.65 ± 0.67	12.96 ± 3.23
Cyclophosphamide	1.75 ± 1.43 <sup>a</sup>	7.35 ± 0.88 <sup>a</sup>	27.26 ± 3.50 <sup>a</sup>
Decoction	1.09 ± 0.58 <sup>#</sup>	10.65 ± 0.93 <sup>#</sup>	32.87 ± 4.23 <sup>#</sup>

<sup>\*</sup>*P* < 0.05, relative to negative control group; <sup>#</sup>*p* < 0.05, relative to cyclophosphamide group; <sup>a</sup>*p* < 0.05, relative to normal control group; n = 10

**RESULTS**

**Basic profiles of mice**

Number of spontaneous activities, tumor inhibition, survival and mean body weight were markedly higher in the decoction group than in negative control and cyclophosphamide groups (*p* < 0.05; Table 1).

**Levels of Tregs in the mice**

As shown in Table 2, serum CD4<sup>+</sup> Th17 level was lower in the decoction group than in negative control and cyclophosphamide groups (*p* < 0.05). However, there were significantly higher serum CD4<sup>+</sup>CD25<sup>+</sup> Treg and Th17/Treg ratio in the decoction group than in the control and cyclophosphamide groups (*p* < 0.05).

**TNF-α and IL-2 levels**

As shown in Table 3, serum TNF-α and IL-2 levels were markedly higher in the decoction group than in negative control and cyclophosphamide groups (*p* < 0.05).

**Table 3:** Levels of TNF-α and IL-2 (ng/L)

Group	TNF-α	IL-2
Normal control	123.23 ± 42.15	105.25 ± 29.56
Negative control	132.21 ± 47.23	108.32 ± 31.25
Cyclophosphamide	82.15 ± 15.36	75.36 ± 25.64
Decoction	299.56 ± 45.23 <sup>#</sup>	279.56 ± 59.23 <sup>#</sup>

<sup>\*</sup>*P* < 0.05, relative to negative control group; <sup>#</sup>*p* < 0.05, relative to cyclophosphamide group (n = 10)

### Serum levels of P21 protein and VEGF

Table 4 shows significantly lower serum levels of P21 protein and VEGF in decoction group than in negative control and cyclophosphamide groups. These parameters were markedly lower in cyclophosphamide group than in negative control group ( $p < 0.05$ ). However, serum P21 protein and VEGF levels were comparable in the decoction and normal control groups ( $p > 0.05$ ).

**Table 4:** P21 protein and VEGF levels in serum

Group	Serum P21 protein (pg/L)	VEGF ( $\mu$ L)
Normal control	25.36 $\pm$ 2.87	1.23 $\pm$ 0.45
Negative control	32.13 $\pm$ 4.12	3.38 $\pm$ 0.47
Cyclophosphamide	28.26 $\pm$ 3.80 <sup>a</sup>	1.78 $\pm$ 0.40 <sup>a</sup>
Decoction	26.03 $\pm$ 2.65 <sup>#</sup>	1.28 $\pm$ 0.73 <sup>#</sup>

<sup>#</sup> $p < 0.05$ , relative to negative control group; <sup>a</sup> $p < 0.05$ , relative to cyclophosphamide group; <sup>a</sup> $p < 0.05$ , relative to the normal control group;  $n = 10$

### Thymus and spleen indices

Thymus and spleen indices were significantly elevated in the decoction group, when compared with the negative control and cyclophosphamide groups ( $p < 0.05$ ), but were not significantly different from those of normal control group ( $p > 0.05$ ; Table 5).

**Table 5:** Thymus and spleen indices

Group	Thymus index	Spleen index
Normal control	2.34 $\pm$ 0.48	8.61 $\pm$ 0.75
Negative control	1.62 $\pm$ 0.23 <sup>†</sup>	5.63 $\pm$ 2.42 <sup>†</sup>
Cyclophosphamide	1.73 $\pm$ 0.48 <sup>†</sup>	5.87 $\pm$ 1.65 <sup>†</sup>
Decoction	2.35 $\pm$ 0.45 <sup>#</sup>	7.54 $\pm$ 3.95 <sup>#</sup>

<sup>†</sup> $P < 0.05$ , relative to negative control group; <sup>#</sup> $p < 0.05$ , relative to cyclophosphamide group;  $n = 10$

## DISCUSSION

The pathogenesis of LC is quite complicated, and is characterized by spleen and stomach weaknesses, sputum qi, blood deficiencies, eating disorders, qi stagnation and blood stasis. Liver damage induced by different factors results in Zheng qi weakness and poisonous phlegm and ultimately lead to LC [6,7]. Radiotherapy and chemotherapy kill tumor cells and also cause some degree of damage to normal cells. Therapeutics used in TCM significantly improve liver function and reduce incidents of adverse reactions. Therefore, the combination of Chinese medicine and surgery after chemotherapy constitutes an effective treatment strategy for LC.

This strategy involves detoxification, clearing of heat, enhancement of blood circulation, and removal of blood stasis, amongst others [8].

In the present study, tumor inhibition, survival, body weight and the number of spontaneous activities were appreciably enhanced in the decoction, relative to the negative control and cyclophosphamide groups. These results suggest that *Yugan Sanjie* decoction may effectively mitigate the clinical symptoms of LC and inhibit proliferation of the tumor cells. It is possible that constituents of the decoction contributed synergistically to the observed effects. *Isatidis radix*, *Astragalus*, *Bupleurum*, and *Paeonia albiflora* Pall enhance spleen function; *Salvia miltiorrhiza* regulates pain, while tangerine promotes blood circulation and joint function [9].

Cellular immunity is an important defense mechanism against tumor cells, and T lymphocytes are involved in the regulation of immune function. The CD4<sup>+</sup> T lymphocytes are the dominant cells of cellular immune system, and they differentiate into lymphocyte subsets such as T-helper cell 17 (Th17) and Treg. In turn, Th17 stimulates neutral cells and macrophages to secrete the inflammatory cytokine IL-6, thereby aggravating the inflammatory response. It is known that Treg inhibits the inflammatory response via the secretion of IL-8 and other factors which help to maintain stability of the autoimmune system.

Studies have shown that the incidence of many tumors are closely linked to the imbalance between the levels of Th17 and Treg. Dynamic balance between Th17 and Treg is important for prevention of the inflammatory response and maintenance of immune system stability [10,11]. Tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), a small protein molecule secreted by macrophages directly kills and inhibits tumor cells, and it is presently recognized as the most potent anti-tumor cytokine. Interleukin-2 (IL-2) participates in humoral immunity and exerts an important immune function via the stimulation and activation of T cells. Studies have shown that TNF- $\alpha$  and IL-2 levels are significantly lower in peripheral blood of patients with LC than in normal patients [12].

In this study, serum level of CD4<sup>+</sup> Th17 was markedly low in the decoction group, relative to the negative control and cyclophosphamide groups. However, serum level of CD4<sup>+</sup>CD25<sup>+</sup> Treg and Th17/Treg were appreciably elevated in decoction group, when compared to the negative control and cyclophosphamide groups. Serum

levels of TNF- $\alpha$  and IL-2 were markedly higher in decoction group than in negative control and cyclophosphamide groups. These results suggest that decoction of *Yugan Sanjie* may effectively improve immune function in mice. It is possible that *Yugan Sanjie* decoction inhibited the tumor via the regulation of neutral cells and macrophages, leading to the release of antigenic and other soluble factors which stimulated proliferation of natural killer (NK) cells. It is also possible that the decoction stimulated NK cell killing activity, induced interferon (IFN) secretion and activated macrophages which in turn killed tumor cells [13].

Studies have shown that serum P21 protein promotes the proliferation and differentiation of tumor cells by regulating signals related to the cells, and it is highly expressed in LC cells. The most effective regulator of tumor angiogenesis is VEGF, and it promotes the growth of vascular endothelial cells, increases permeability, growth and metastasis of tumor cells. Therefore, the inhibition of serum P21 protein and VEGF expression is important for inhibiting tumor growth [14].

In this study, serum levels of P21 protein and VEGF were significantly lower in decoction group than in negative control and cyclophosphamide groups, and were significantly lower in cyclophosphamide group than in negative control group. However, there were no significant differences in serum levels of P21 protein and VEGF between decoction and normal control groups. These results appear to suggest that decoction of *Yugan Sanjie* may inhibit tumor cell proliferation and invasion through various mechanisms, and are in agreement with those previously reported [15].

The thymus and spleen are two important immune organs, and their indices reflect the level of cell immunity. These indices were significantly higher in the decoction group than in the negative control and cyclophosphamide groups, but were comparable with those of the normal control group. These results appear to suggest that *Yugan Sanjie* decoction may confer some level of protection on immune organs, and thus may inhibit tumor proliferation. These findings are consistent with those reported previously [16].

## CONCLUSION

Decoction of *Yugan Sanjie* effectively alleviates the symptoms of LC and improves immune function of mice by regulating serum levels of T lymphocytes. It inhibits tumor angiogenesis by

suppressing the expressions of serum P21 protein and VEGF. These findings provide scientific evidence in support of the use of *Yugan Sanjie* decoction as a new treatment strategy for LC.

## DECLARATIONS

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### Conflict of interest

No conflict of interest is associated with this work.

### Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. Song Tan conceived and designed the study; Song Tan, Aihua Hou, Wei Liu, Jinguo Wang, Xiaoming Huang and Yuejun Mu collected and analysed the data, while Yuejun Mu wrote the manuscript.

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