[Briefing]

# Studies on the biological behavior and mechanism of human hepatocellular carcinoma cells SMMC-7721 by the traditional Chinese medicine ZYD of anti-hepatitis B virus

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**Abstract:**

**Objective** To evaluate the effect of anti-hepatitis B virus traditional Chinese medicine ZYD on the biological behavior of human hepatocellular carcinoma cells SMMC-7721, and to discuss their possible mechanism. Methods The cell proliferation was detected by MTT; the cell adhesion ability was detected according to cell adhesion experiment; the cell migration ability was determined by scratch test; the cell cycle and apoptosis were tested by flow cytometry, and the reverse-transcription-polymerase chain reaction (RT-PCR) was applied to detect the MMP-2、MMP-9, TIMP-1, Snail, Vimentin, and Prx-1 mRNA expression. **Results** ZYD could significantly inhibit the proliferation of SMMC-7721 cells, and the inhibitory effect was better than each herb component. ZYD could inhibit the adhesion of SMMC-7721 cells in a dose-dependent manner, and could reduce the migratory ability of SMMC-7721 cells by dose-effect and time-effect manner. ZYD could promote apoptosis of SMMC-7721 cells and lead to cell cycle block in S stage. Furthermore, the MMP-2 and Snail mRNA expression in SMMC-7721 cells was significantly down-regulated at each dose, while high dose (1 000 μg/ml) of ZYD could significantly reduce MMP-9 mRNA expression. Conclusion ZYD can inhibit the proliferation of SMMC-7721 hepatoma cells by promoting cellular apoptosis and blocking cell cycle in S stage. Moreover, ZYD can suppress the metastasis of hepatocellular carcinoma cells by decreasing Snail, MMP-2 and MMP-9 mRNA expression.

**Key words:** traditional Chinese medicine ZYD; hepatocellular carcinoma; SMMC-7721 cell; proliferation; metastasis

**CLC:** R735.2; R932 **Document code:** A **Article ID:** 1672-9188(2018)05-0306-07

DOI：10.13683/j.wph.2018.05.005

Receiving date: February 3, 2018; Revision date: March 6, 2018

Brief introduction of author: Guo Ping, Researcher, Research direction: Basic and clinical research on treatment of metabolic disorders by traditional Chinese medicine, Major in: Research and development of new drugs, evaluation on in vitro and in vivo efficacies and study on corresponding mechanisms.

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Fund program: Innovation Fund for Middle and Small-sized Enterprises (Code: 09C26211100043).

Hepatocellular carcinoma is one of the most common malignant tumors, of which, the mortality ranks third in the world for cancers and ranks second in China [1], and is characterized as asymptomatic on early onset of occurrence, high grade malignancy, rapid disease progress, short survival and high fatality rate, etc. The theory from traditional Chinese medicine mainly categories the hepatic carcinoma in the range of "abdominal mass, amassment and accumulation, tympanites, jaundice, hypochondriac pain, gallbladder distention, distention and fullness as well as cancer", of which, the main cause is dysfunction spleen due to its injury resulting from overstrain, or liver dysfunction resulting from emotional depression, resulting in various pathological conditions such as blood stasis, toxin, phlegm, damp and fever produced in the body [2].

The anti-hepatitis B virus traditional Chinese medicine ZYD is made of components such as phyllanthus urinaria L., radix salviae miltiorrhizae, coriolus versicolor mushroom and radix arnebiae which play a role in anti-tumors [3-6], and is indicated for treatment of patients with chronic viral hepatitis B (with syndrome differentiation of internal retention of damp-heat and blood stasis due to qi deficiency). The hepatitis B surface antigens (HBsAg) test results in 80%-95% patients with hepatocellular carcinoma in our country present positive [7], the hepatitis B virus infection has been recognized as the main cause of hepatocellular carcinoma, and if corresponding therapy is not given appropriately, the persistent injury of liver will develop to cirrhosis so as to potentially give rise to nodules of hepatocellular carcinoma [8], which also provide a theoretical basis and clue for treatment of hepatocellular carcinoma by the traditional Chinese medicine ZYD. This study was designed to observe the change of biological behavior of hepatocellular carcinoma cells SMMC-7721 under pharmacological intervention, to evaluate the in vitro anti-tumor effect of traditional Chinese medicine ZYD, to discuss its possible mechanism of action in treatment of hepatocellular carcinoma and to evaluate the differences of efficacy between traditional Chinese medicine ZYD and each herb component so as to provide references for treatment of hepatocellular carcinoma by traditional Chinese medicine.

## 1. Materials and methods

### 1.1 Cells and drugs

The human hepatocellular carcinoma cell strain SMMC-7721 was provided by Shanghai Cell Bank of Chinese Academy of Sciences. ZYD (Manufacturing batch no.: 13040102) was a mixture of extracts of different components, the water extract of phyllanthus urinaria L. filtered with macroporous resin, and the alcohol extract of phyllanthus urinaria L., the water extract of phyllanthus urinaria L., the water extract of coriolus versicolor mushroom, and the alcohol extracts as well as water extract of radix arnebiae and radix salviae miltiorrhizae were purchased from Beijing Han Dian Pharmaceutical Co., Ltd.

### 1.2 Methods

1.2.1 Effect of ZYD and its single herb on proliferation of SMMC-7721 cells were detected by methyl thiazolyl tetrazolium colorimetric method (MTT)

The human hepatocellular carcinoma cell strain SMMC-7721 was routinely cultivated in 1640 complete culture medium containing 10% FBS, and $3\times10^3$ cells was inoculated to each well of a 96-well plate. Different test samples at concentrations of 500μg/ml and 1000 μg/ml were added: ZYD (finished product group), mixture of components of ZYD (full component

group), phyllanthus urinaria L. filtered with macroporous resin (component group 1), alcohol extract of phyllanthus urinaria L (component group 2), water extract of phyllanthus urinaria L (component group 3), water extract of coriolus versicolor mushroom (component group 4), alcohol extracts of radix arnebiae and radix salviae miltiorrhizae (component group 5), and water extracts of radix arnebiae and radix salviae miltiorrhizae (component group 6). Each sample was added into 3 parallel wells and cultivated for 24, 48 and 72 h for MTT assay. Inhibition rate (%) $=(A_{blank}-A_{measurement})/(A_{blank}-A_{zeroing}) \times 100\%$.

1.2.2 The cell adhesion ability SMMC-7721 cells was detected according to cell adhesion experiment

$1\times10^6$ cells were inoculated to each well of a 6 well-plate, and ZYD at concentrations of 500, 750, 1000, 1500 and 2000 μg/ml was added into 3 parallel wells, and after drug action for 48 h, the cells were collected and re-suspended in 1640 complete medium containing 10% FBS and then were inoculated to a 24-well plate for incubation for 4 h. The cells were fixed for 20 min with methyl alcohol (or for 10 min with 95% alcohol), stained with 0.1% crystal violet for 15-20 min, washed for 3 times with clean water, and then dried and photographed. The inhibition rate of cell adhesion (%) =(Quantity of cell adhesion in the control group - Quantity of cell adhesion in the component group)/Quantity of cell adhesion in the control group×100%.

1.2.3 Migration ability of SMMC-7721 cells was determined by scratch test

$1\times10^6$ cells were inoculated to each well of a 6-well plate, and ZYD at concentrations of 500, 1000, 1500 and 2000 μg/ml was added into 3 parallel wells. After incubation where the culture plate was covered with cells, the supernatant was discarded, marked a "-" letter on the 6-well plate with a tip of 20 μl and complete medium was added to the control group. The cells were photographed on 0, 24 and 48 h after drug action under 100 X inverted microscope. The healing rate (%) = (Width of trace before addition of drugs-Width of trace after addition of drugs)/Width of trace before addition of drugs×100%.

1.2.4 Cell apoptosis and cell cycle of SMMC-7721cells were tested by flow cytometry $1\times10^6$ cells were inoculated to a 6 well-plate, and ZYD at concentrations of 1500, 2000, 2500, 3000, 4000, 500, 750 and 1000 μg/ml was added and cultivated for 48 h at 37℃. The cell apoptosis and cell cycle of SMMC-7721 cells were tested by flow cytometry.

1.2.5 Reverse-transcriptase-polymerase chain reaction (RT-PCR) was applied to detect the expression of invasion and metastasis related genes of SMMC-7721 cells

$1\times10^6$ cells were inoculated to each well of a 6-well plate and ZYD at concentrations of 500, 1000 and 2000 μg/ml were added and cultivated for 48 h. The total RNAs were extracted by Trizol method, the genes of *Snail* (3'-CCTTCGTCCTTCTCCTCTACTT and 5'-GCTTCTTGACATCTGAGTGGGT: 94℃ for 2 min, 94℃ for 30s，56.4℃ for 30 and 72℃ for 60s, 35 cycles, and then 72℃ for 5 min), *Vimentin* (3'-CGA CGC CAT CAA CAC CGA GT and 5'-CCG TGA GGT CAG GCT TGG AA: 94℃ for 2 min, 94℃ for 30s, 56.4℃ for 30s, 72℃ for 60s, 26 cycles and then 72℃ for 5 min), *Peroxidase-1* (Prx-1) (3'-AGGAAATGCTAAAATTGGGCACC and 5'-TCTTTGCTCTTTTGGACATCAGG; 94℃ for 2 min, 94℃ for 30 s, 59.7℃ for 30 s, 72℃ for 60 s, 35 cycles and then 72℃ for 5 min), *Matrix Metalloproteinase (MMP)-2* (3'-GGATGATGCCTTTGCTCG and 5'-CAGTGGACATGGCGGTCT; 94℃ for 2 min, 94℃ for 30 s, 61℃ for 30 s, 72℃ for 60 s, 40 cycles and then 72℃ for 5 min), *MMP-9* (3'-GGCTACGTGACCTATGACATCCT and 5'-TCCTCCCTTTCCTCCAGAACA; 94℃ for 2 min, 94℃ for 45 s, 60.5℃ for 45 s, 72℃ for 45 s, 40 cycles and then 72℃ for 5 min), *Tissue Inhibitor of Metalloproteinase-1 (TIMP-1)* (3'-CCAGAGAGACACCAGAGAACC and 5'-GCTGGTATAAGGTGGTCTGGT; 94℃ for 2

min, 94℃ for 30 s, 60.3℃ for 30 s, 72℃ for 60 s, 35 cycles and then 72℃ for 5 min), and *β-actin* (3'-TGACGTGGACATCCGCAAAG and 5'-CTGGAAGGTGGACAGCGAGG; 94℃ for 2 min, 94℃ for 30 s, 58℃ for 30 s, 72℃ for 60 s, 30 cycles and then 72℃ for 5 min) were amplified; The PCR products were analyzed by agarose gel electrophoresis.

### 1.3 Statistical methods

SPSS 17.0 software was used for statistical analysis on data. The measurement data was expressed as mean ± SD ($\bar{x}\pm s$), the measurement data of multiple groups were analyzed by One way ANOVA, LSD was used for the multiple comparisons , the linear correlation was analyzed by linear regression and P<0.05 meant that the difference was statistical significant.

## 2. Results

2.1 Effect on the proliferation of SMMC-7721 cells of ZYD and its single herb

As shown in Table 1, with extension of action duration and increase of administration dose, except the component group 4, all other groups showed a gradual inhibition in proliferation of SMMC-7721 cells, and the inhibition rate was finished product group ＞ full component group ＞ each herb component by dose-effect and time-effect manner. At dose of 500 μg/ml, the inhibition rates at 48 h and 72 h in finished product group and full component group were better than that in cell control group (P<0.01), and the inhibition rates at 72 h in component groups 2, 3 and 5 were better than that in cell control group (P<0.05 or P<0.01); At dose of 1000 μg/ml, the inhibition rates at 24 h, 48 h and 72 h in finished product group and full component group were better than that in cell control group (P<0.01), and the inhibition rates at 72 h in component groups 1, 2, 3, 5 and 6 were better than that in cell control group (P<0.01).

2.2 Effect on the cell adhesion ability of SMMC-7721 cells of ZYD

Except the 500 μg/ml group of ZYD, compared with cell control group, the cell quantities in other dose groups reduced (P<0.01), which was decreased progressively with increasing concentration of ZYD. See Table 2.

2.3 Effect on the cell migration ability of SMMC-7721 cells of ZYD

As shown in Table 3, the healing rates in various dose groups of ZYD were decreased progressively with increasing dose and were increased with increasing dosing time. After 24 h of scratching, the healing rate in cell control group was 85.5%, and the healing rates in each dose group of ZYD were lower than that in cell control group (P<0.05 or P<0.01); After 48 h of scratching, the scratch in cell control group completely healed, and the healing rates in each dose group of ZYD were lower than that in cell control group with statistical significant difference (P<0.01).

Table 1 Effect on the proliferation of SMMC-7721 cells of ZYD and its single herb ($\bar{x}\pm s$, n=3)

| Group | Inhibition rate (%) | | |
|---|---|---|---|
| | 24 h | 48 h | 72 h |
| Cell control group | 0 | 0 | 0 |
| Finished product group (500 μg/ml) | -13.3±10.4** | 19.5±4.1** | 45.4±1.8** |
| Full component group (500 μg/ml) | -6.8±2.3 | 6.7±2.1** | 24.7±2.3** |
| Component group 1 (500 μg/ml) | -10.4±5.6* | -0.7±2.6 | 1.9±0.8 |

| Group | Inhibition rate (%) | | |
|---|---|---|---|
| | 24 h | 48 h | 72 h |
| Component group 2 (500 μg/ml) | -11.1±1.3* | -3±2.9 | 7.1±3.0** |
| Component group 3 (500 μg/ml) | -24.6±6.7** | -5.3±2.8* | 4.6±3.7* |
| Component group 4 (500 μg/ml) | -29.4±5.5** | -21.1±0.3** | -14.8±2.9* |
| Component group 5 (500 μg/ml) | -6.5±3.3 | -0.8±4.9 | 7.1±3.0** |
| Component group 6 (500 μg/ml) | -12.1±3.5* | -12.8±0.6** | -3.3±1.2 |
| Finished product group (1000 μg/ml) | 17.6±3.9** | 54±6.3** | 76.9±3.3** |
| Full component group (1000 μg/ml) | 17.8±2.6** | 40.1±5.3** | 70.2±3.1** |
| Component group 1 (1000 μg/ml) | -12.5±4.2** | 7.9±4.0 | 22.5±5.5** |
| Component group 2 (1000 μg/ml) | -17.3±1.6** | -1±3.1 | 19.3±2.6** |
| Component group 3 (1000 μg/ml) | -21±3.7** | 3.4±6.4 | 20.2±7.3** |
| Component group 4 (1000 μg/ml) | -36.1±3.6** | -22.9±6.3** | -3.4±4.4 |
| Component group 5 (1000 μg/ml) | -10.6±3.9** | 7.1±3.8 | 16.5±3.9** |
| Component group 6 (1000 μg/ml) | -22.1±4.9** | -6.2±2.0 | 10.9±4.2** |

vs the cell control group, *P＜0.05, **P＜0.01

Table 2 Effect on the cell adhesion ability of SMMC-7721 cells of ZYD ($\bar{x}\pm s$, n=3)

| Group | Cell quantity/cells | Inhibition rate (%) |
|---|---|---|
| Cell control group | 396±28 | |
| ZYD groups | | |
| 500 μg/ml | 367±27 | 7.3 |
| 750 μg/ml | 227±13** | 42.7 |
| 1000 μg/ml | 93±7** | 76.5 |
| 1500 μg/ml | 46±5** | 88.4 |
| 2000 μg/ml | 5±2** | 98.7 |

vs the cell control group, **P＜0.01

Table 3 Effect on the cell migration ability of SMMC-7721 cells of ZYD ($\bar{x}\pm s$, n=3)

| Group | Healing rate (%) | |
|---|---|---|
| | 24 h | 48 h |
| Cell control group | 85.5±2.6 | 100.0±0.0 |
| ZYD groups | | |
| 500 μg/ml | 78.9±4.5* | 86.6±1.5** |
| 1000 μg/ml | 62.3±2.7** | 69.2±1.8** |
| 1500 μg/ml | 52.9±2.6** | 65.4±1.3** |
| 2000 μg/ml | 26.8±6.7** | 41.7±4.3** |

vs the cell control group, *P＜0.05, **P＜0.01

Table 4 Effect on the apoptosis of SMMC-7721 cells of ZYD ($\bar{x}\pm s$, n=3)

| Group | Cell apoptosis rate (%) |
|---|---|
| Cell control group | 4.2±0.4 |
| ZYD groups | |
| 1500 μg/ml | 45.7±4.3** |
| 2000 μg/ml | 50.2±3.7** |
| 2300 μg/ml | 52.1±5.6** |

| Group | Cell apoptosis rate (%) |
|---|---|
| 3000 μg/ml | 56.2±5.0** |
| 4000 μg/ml | 88.6±6.7** |

vs the cell control group, **P＜0.01

Table 5 Effect on the cell cycle of SMMC-7721 cells of ZYD ($\bar{x}\pm s$, n=5)

| Group | Cell cycle (%) | | |
|---|---|---|---|
| | G0/G1 stage | S stage | G2/M stage |
| Cell control group | 75.2±5.2 | 18.3±2.1 | 6.5±1.6 |
| ZYD groups | | | |
| 500 μg/ml | 65.1±4.4 | 32.7±2.3* | 2.2±0.8 |
| 750 μg/ml | 56.3±4.1 | 41.2±3.3** | 2.5±1.2 |
| 1 000 μg/ml | 56.2±5.6 | 42.6±3.9** | 1.1±1.8 |

vs the cell control group, *P＜0.05, **P＜0.01

2.4 Effect on the apoptosis and the cell cycle of SMMC-7721 cells of ZYD

The cell apoptosis test results showed that after incubation for 48 h, compared with cell control group, the cell apoptosis rates of ZYD at each dose group increased and the differences had statistical significance (P<0.01), and the apoptosis rates increased with increase of administration dose (Table 4).

The cell cycle test results showed that after incubation for 48 h, compared with cell control group, the proportion of cell quantity in S stage of ZYD at each dose group increased and the differences had statistical significance (P<0.05 or P<0.01), and there was no statistical significant difference between the proportion of cell quantities in G0/G1 stage and G2/M stage (P>0.05) (Table 5).

2.5 Effect on the expression of invasion and metastasis related genes of SMMC-7721 cells of ZYD

Compared with the cell control group, the *MMP-2* (P<0.05 or P<0.01) and *Snail* (P<0.01) mRNA expression in SMMC-7721 cells of ZYD at each dose group was decreased, and *MMP-9* mRNA expression was down-regulated, of which, there was a statistically significant difference between the expressions in the ZYD (2,000 μg/ml group) and cell control group (P<0.01). There were no statistically significant differences of *TIMP-1*, *Vimentin* and *Prx-1* mRNA expression in SMMC-7721 cells between ZYD at each dose group and cell control group (P>0.05) (Figure 1 and Table 6).

## 3. Discussion

Hepatocellular carcinoma is a disease seriously threatening human health and is routinely treated by surgery, intervention and chemotherapy, etc. in clinical practice. The drugs targeting on hepatocellular carcinoma such as Sorafenib also has toxic and side effects while extending survival of patients [9]. Moreover, some drugs such as doxorubicin and fluorouracil are not effective and have large toxic and side effects, no matter used alone or in combination. Thus, development of anti-hepatoma drugs with lower toxicity has become a hot topic and challenge in clinical studies. The traditional Chinese medicines that are inherited for thousands of years in our

country have obvious advantages in safety, and there are various kinds of Chinese patent medicines for treatment of hepatocellular carcinoma such as GFL tablet with certain efficacy and less toxic and side effects are mostly used as adjuvant treatment medications for hepatocellular carcinoma.

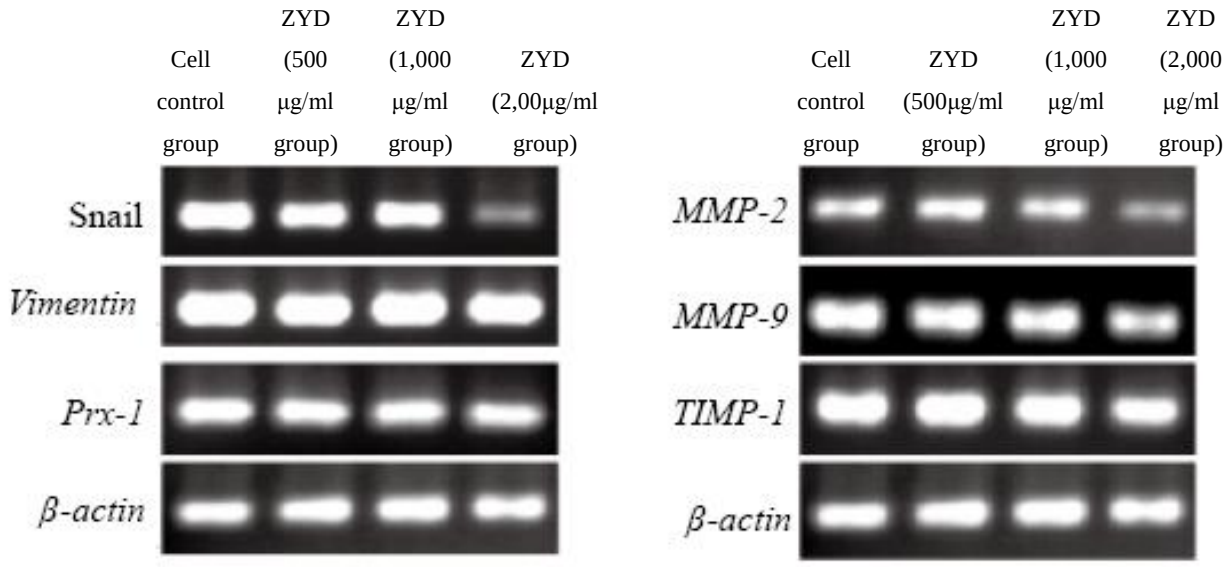


**Figure 1 Electrophorogram on the invasion and metastasis related genes of SMMC-7721 cells of ZYD**

**Table 6 Effect on the invasion and metastasis related genes of SMMC-7721 cells of ZYD ($\bar{x}\pm s$, n=3)**

| Group | Relative expressions of genes | | | | | |
|---|---|---|---|---|---|---|
| | *Snail* | *Vimentin* | *Prx-1* | *MMP-2* | *MMP-9* | *TIMP-1* |
| Cell control group | 1.10±0.05 | 1.75±0.07 | 1.22±0.06 | 1.03±0.09 | 1.19±0.07 | 1.39±0.05 |
| ZYD groups | | | | | | |
| 500 μg/ml | 0.91±0.05** | 1.68±0.19 | 1.11±0.11 | 0.86±0.09* | 1.12±0.12 | 1.33±0.08 |
| 1 000 μg/ml | 0.90±0.04** | 1.71±0.19 | 1.15±0.06 | 0.84±0.08* | 1.06±0.09 | 1.35±0.09 |
| 2 000 μg/ml | 0.76±0.08** | 1.64±0.21 | 1.12±0.04 | 0.70±0.09** | 0.94±0.06** | 1.24±0.05 |

vs the cell control group, *P＜0.05, **P＜0.01

The traditional Chinese medicine ZYD has an advantage of low toxic and side effects and an effect on anti-hepatitis B viruses, and is indicated for high carrying rate of hepatitis B viruses in patients with hepatocellular carcinoma in our country, which was rare in Chinese patent medicines for hepatocellular carcinoma. The component of phyllanthus urinaria L. is used as a sovereign drug having sweet-bitter nature and flavor and being cool-natured, enters liver and lung channel for suppressing hyperactive liver and clearing heat as well as inducing diuresis for detoxication; Coriolus versicolor mushroom is used as a minister drug having sweet flavor and being neutral in nature, can benefit qi and nourishing yin, strengthen vital qi to eliminate pathogenic factor to coordinate yin and yang; The sovereign drug eliminates pathogenic factor by detoxification, the minister drug strengthens vital qi by invigorating yin, and the combination plays a role in eliminating pathogenic factor and strengthening vital qi. Radix salviae

miltiorrhizae is used as an assistant and envoy drug having bitter flavor and being slightly cold-natured, enters heart and liver channel for clearing nutrient level and cooling blood, and promoting blood circulation for removing obstruction in collaterals, and is used for promoting blood circulation to remove blood stasis and removing edema to relieve pain; Radix arnebiae is also used as an assistant and envoy drug having bitter salty flavor and being cold-natured, enters heart and liver channel for cooling blood, promoting blood circulation and detoxification, and also can play a role in clearing heat, cooling blood and detoxification as well as enhancing in promoting blood circulation to remove blood stasis when used in combination with phyllanthus urinaria L.

The pathogenesis of carcinoma is very complicated and is caused by multiple factors, many steps and mutations of many genes, which mainly presents as malignant proliferation and disorder apoptosis of cells, and in other words, the malignant proliferation of cells is the basis of tumorigenesis and effective inhibition of proliferation of tumor cells is the first step for studying anti-tumor drugs [10]. This study observed the effect of ZYD and extracts of each herb component on SMMC-7721 cells by MTT method, and the results demonstrated that the inhibition of proliferation of SMMC-7721 cells was in the order of ZYD ＞ mixture of extracts＞ each herb component by dose-effect and time-effect manner, which indicated that the ZYD had a role in inhibiting SMMC-7721 cells and also validated the rationality of the formula of the compound. The further test results of cell apoptosis and cell cycle indicated that after incubation for 48 h, ZYD at dose of above 1500 μg/ml could induce the apoptosis of SMMC-7721 cells and ZYD at doses of 500-1000 μg/ml could induce cell cycle block of SMMC-7721 cells in S stage. All the results suggested that ZYD could inhibit the proliferation of SMMC-7721 cells by inducing apoptosis of SMMC-7721 cells and inhibiting cell cycle block in S stage.

The tumor metastasis is a complicated process. The tumor cells would fall off the primary tumor and infiltrate into the basement membrane epithelium and epithelium, so as to enter the circulating system, and spread and then adhere to other sites so as to proliferate and form a metastatic tumor [11], therefore, the metastatic capacity of a tumor depends on the abilities of proliferation, adhesion, migration and invasion of tumor cells. The epithelial-mesenchymal transition (EMT) is an important biological process during which the malignant cells derived from epithelial cells acquire capabilities of migration and invasion. E-cadherin belongs to type I cadherin, which is connected to the actin cytoskeleton by α proteins and β proteins in the cytoplasm to form an extracellular immunoglobulin domain so as to form a stable cell-cell contact and block cytolergy invasion and metastasis [12]. Snail is a zinc finger protein, which can identify the E box in the promoter region of E-cadherin by DNA binding factor, inhibit the expression of E-cadherin and induce occurrence of EMT. Vimentin is an intermediate filament protein found in the mesenchymal cells, which is closely related to tumorigenesis and tumor metastasis, and it can regulate the interaction among proteins such as cytoskeletal proteins and cell adhesion molecules, involve in adhesion, migration, invasion and signal transduction of tumor cells as well as endothelial cells and macrophages related to tumors [13]. Some studies showed that increased expression of molecular indicator *Vimentin* of the interstitial phenotype could promote the occurrence of EMT. As shown from adhesion and scratch test results in this study, ZYD could inhibit the adhesion and migratory ability of SMMC-7721 cells by dose-effect and time-effect manner. And the RT-PCR test results showed that ZYD could down-regulate *Snail* mRNA expression in SMMC-7721 cells while did not significantly impact *Vimentin* mRNA expression, which indicated that the action of mechanism that ZYD inhibited metastasis

of hepatocellular carcinoma might be correlated to down-regulation of *Snail* mRNA expression.

Prx 1 is a member of the Prx protein family, and the latter is a series of newly discovered peroxidases which have strong capabilities in antioxidation and clearing free radicals, are highly expressed in multiple malignant tumors and are correlated to occurrence, development, invasion and metastasis of tumors as well as sensitivity to chemoradiotherapy [14]. This study result indicated that ZYD did not impact *Prx 1* mRNA expression, thus the action of mechanism for metastasis of hepatocellular carcinoma should be not related to *Prx 1*.

The Type IV Collagen is a main structural protein involved in ECM and of basilar membrane, while MMP contains MMP-2 and MMP-9 and is the only enzyme for degrading the triple helix site of Type IV Collagen, thus, the overexpression of MMP significantly contributes to invasion and metastasis of tumors [15]. The expression and activity of MMP are regulated by transcriptional level, activation of zymogen and inhibition effects of inhibitors [16]. TIMP-1 is a glycoprotein with molecular weight of 28.5 kDa, can inhibit most MMPs and form a non-covalent complex of high affinity with the precursor of MMP-9 or activated MMP-9 so as to block the activation of MMP-9 or directly inhibit its activity [17]. A great number of studies demonstrated that the up-regulated activity and expression of MMP as well as down-regulated activity and expression of TIMP-1 broke the balance between MMP-9 and TIMP-1, increasing invasion and metastasis abilities of malignant tumors. From the RC-PCR results in this study, ZYD could reduce *MMP-2* and *MMP-9* mRNA expression, while did not increase *TIMP-1* expression, extrapolating that ZYD might regulate *MMP-2* and *MMP-9* mRNA expression by other signal paths.

## 4. Conclusions

In conclusion, the traditional Chinese medicine ZYD could inhibit the proliferation of SMMC-7721 hepatoma cells by promoting cell apoptosis and blocking cell cycle in S stage, while can suppress the metastasis of hepatocellular carcinoma cells by decreasing *Snail*, *MMP-2* and *MMP-9* mRNA expression.

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(Editor in charge: Zhao Xutao)