Cidan inhibits liver cancer cell growth by reducing COX-2 and VEGF expression and cell cycle arrest

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Abstract. Cidan is a traditional Chinese medicine formula that has been used for >10 years as an antitumor drug. In the present study, the antitumor effect of cidan on hepatocellular carcinoma (HCC) and the underlying molecular mechanisms were investigated. A total of 372 patients with primary HCC, as confirmed by pathological examination in the Eastern Hepatobiliary Surgery Hospital and Beijing Oncology Hospital of Weida TCM, were prospectively enrolled in the study. In total, 92 patients were treated with cidan capsules for three months postoperatively, while 280 patients served as controls. The efficacy of cidan was analyzed by monitoring associated symptoms and liver function tests, including measuring the levels of α-1-fetoprotein, α-L-fucosidase, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase and γ-glutamyl transferase. In addition, *in vivo* analysis was performed using mice Hepa1-6 xenograft models, while *in vitro* studies were performed with SMMC-7721 and CSQT-1 cells; this included cidan-dependent cell viability and migration assays, cell cycle analyses and the evaluation of cidan effects on cyclooxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF) mRNA transcription rates using quantitative polymerase chain reaction. The postoperative two-year overall survival (77 and 58% for the cidan and control groups, respectively; P<0.01) and disease-free survival (36 and 24% for the cidan and control groups, respectively; P<0.01) rates were superior in the cidan-treated group when compared with the control. In addition, the size and weight of the tumor xenografts in the C57BL/6 mice were significantly reduced in a time- and dose-dependent manner following cidan treatment (P<0.01). Cidan significantly reduced the cell viability of SMMC-7721 and CSQT-1 cells after four and five days when compared with the control (P<0.01). Furthermore, COX-2 and VEGF mRNA expression levels decreased following cidan treatment (P<0.01), and cidan treatment resulted in enhanced G1 and G2/M cell cycle arrest of CSQT-1 cells. Therefore, cidan effectively inhibited cell proliferation, reduced cell viability and downregulated COX-2 and VEGF expression levels in hepatoma cells.

Introduction

Primary liver cancer, also known as hepatocellular carcinoma (HCC), is the third leading cause of cancer-associated mortalities worldwide with ~600,000 cases reported annually (1). Chronic liver diseases, viral hepatitis and alcoholism, as well as dietary carcinogens, including aflatoxins and nitrosoamines, are known etiologies (2). In China, the incidence rate of HCC is particularly high due to the large number of patients with cirrhosis caused by chronic hepatitis following viral hepatitis B and C infections (3-5). Postoperative HCC recurrence rates are as high as 50% (6) and at present, therapies primarily include surgery, local ablation, interventional and radiation therapies, in addition to medication. Superior curative effects may be achieved for HCC treatments when chemotherapy is combined with interventional traditional Chinese medicine (TCM), including Curcuma oil (microsphere) perfusion embolization. This intubation technique exhibited similar effects to western medicine using hepatic arterial transcatheter chemoembolization with regard to tumor size and survival time, but was also found to have a less severe effect on peripheral white blood cells and liver damage (7). A variety of TCMs combined with interventional hepatic artery chemotherapy embolization for toxicity reduction and enhancing the efficacy have been investigated for the treatment of HCC. There are two main advantages for TCM treatments of HCC: Firstly, the cytotoxic effects of the ingredients contained in the active natural anticancer drugs directly inhibit the growth of tumor cells (8-11), and secondly, the improvement of the immune system (12). Cidan capsules are a formula containing more than ten types of plant

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extracts, including Rhizoma Curcumae (19%), *Astragalus* (19.6%), *Cremastra appendiculata* (9.8%), *Salvia miltiorrhiza* (9.8%), hive (9.8%) and *Bombyx batryticatus* (9.8%). Cidan has been clinically used for >10 years as a safe and nontoxic antitumor drug. A number of studies have investigated the clinical application of TCMs for HCC (13,14), demonstrating that β-elemene, which is present in Rhizoma Curcumae and the main component of cidan, may inhibit the proliferation of HepG2 cells in a time- and dose-dependent manner. The results indicated that β-elemene exhibited positive effects on apoptosis and induced the cell cycle arrest of HepG2 cells in the G2/M phase, while Fas and Fas ligand expression levels were markedly increased (15,16). In addition, a meta-analysis demonstrated that β-elemene improved the effect of lung cancer chemotherapy as an adjunctive treatment (14). In the present study, the outcomes of postoperative HCC medications with and without cidan were compared. In addition, the effects of cidan on human HCC cells transplanted in mice were investigated, as well as the effects of cidan on *in vitro* cultivated liver cancer cell proliferation and invasion capabilities and cyclooxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF) expression levels.

Patients and methods

Patients. In total, 372 patients diagnosed with primary liver cancer via surgery and pathological examination were included in the study. A total of 92 patients comprised the cidan group, while the additional 280 patients were controls. The diagnosis and inclusion criteria were based on clinical and pathological observations, which were as follows: (i) AFP ≥400 μg/ml and exclusion of pregnancy, embryonic derived gonad tumors and active or metastatic liver cancer; could feel a swelling, hard, and have large nodular tumor of the liver or clear liver space occupying lesions by imaging examination; (ii) AFP <400 μg/ml and exclusion of pregnancy, embryonic derived gonad tumors and active or metastatic liver cancer, but have the characteristics of liver space-occupying lesions by two types of imaging examinations, or have a positive expression of at least two liver cancer markers (DCP, GGTII, AFU, CAl9-9 or others) and the characteristic of liver space-occupying lesions by one imaging examination; (iii) clear clinical appearance and positive extrahepatic metastasis lesions, including visible hemorrhagic ascites or cancer cells found in the lesion, and exclusion of metastatic liver cancer.

Treatments. The study was prospective, but non-randomized since the treatment group consisted of patients who agreed with the offered cidan treatment. In the treatment group, patients were postoperatively administered 1.35 g cidan capsules (Weida Pharmaceutical Co., Ltd., Beijing, China) three times a day in addition to the conventional liver protective drugs, polyene and phosphatidyl choline, whilst the control group received only conventional liver protective drugs without cidan. Administration of cidan capsules was continued for three months and long-term follow-up was continued with visits every two months. Patients in the two groups received routine treatments, including transarterial chemoembolization, when recurrence occurred. Any other anticancer drugs were discontinued in the treatment process.

Criteria of curative efficacy. Following the tumor resection, the two-year overall survival (OS) and disease-free survival (DFS) times of the patients were monitored. DFS was defined as the number of days from the first day following surgery until tumor recurrence. During the two-year follow-up period, all the patients routinely received general health examinations, blood, urinary and stool analyses, as well as liver and kidney function tests and cardiograms. Efficacy monitoring included: (i) Associated symptoms and signs; (ii) liver function tests analyzing the levels of total bilirubin, direct bilirubin, glutamic-pyruvic transaminase, glutamic oxalacetic transaminase, albumin, prealbumin and bile acid; (iii) enzyme analyses measuring the levels of ALP, GGT and lactic dehydrogenase isoenzyme; (iv) analysis of AFP; and (v) quality of life.

In vivo animal experiment. In total, 90 C57BL/6 mice (age, 5 weeks) were purchased from the Shanghai Laboratory Animal Center (Shanghai, China) and inoculated subcutaneously in the dorsal area with Hepa1-6 cells, diluted to 2×10^6 cells/mouse. After seven days, the mice with cancerous tumors of >5 mm in diameter were divided into three groups, which included the blank control, cidan high-dose (4.80 mg/kg) and cidan low-dose (1.92 mg/kg) groups. The blank control group comprised 30 mice, while the Hepa1-6 inoculation model groups included 10 mice per group. Reagents were infused into the stomach once per day, while the blank control group were administered distilled water. At days 7, 14 and 21 following the start of treatment, the mice were sacrificed by cervical dislocation and the tumors were isolated and measured in order to calculate the growth inhibition ratios. The C57BL/6 mice were housed under specific pathogen-free conditions and animal treatments were conducted in accordance with the Principle of Laboratory Animal Care. The experimental procedures were performed with approval from the Committee of Experimental Animal Administration of the Second Military Medical University Laboratory (Shanghai, China) and written informed consent was provided by all the participating patients at admission.

Cell lines and culture conditions. Hepa1-6 cells were obtained from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology and cultured in high glucose Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml benzylpenicillin and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37°C, as previously described (17). The human hepatoma cell lines, SMMC-7721 and CSQT-1, were established in the laboratory (18). Cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml benzylpenicillin and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37°C. SMMC-7721 and CSQT-1 cells were seeded in 6-cm culture plates and treated with various concentrations of cidan and the saline control (10, 20 and 40 μg/ml) for different time courses. Each measurement was performed with three culture plates.

Cytotoxic activity assay. Cytotoxicity assays were performed according to the MTT method, as previously described (19). MTT was purchased from Sigma-Aldrich (St. Louis, MO, USA). Briefly, the cells were washed twice with phosphate-buffered

saline (PBS) and cultured at a density of 5×10^4 cells/ml in flat-bottomed 96-well microtiter plates in 100 μl RPMI-1640 medium. Several dilutions of the tested compounds, in 100 μl RPMI-1640 medium with 10% FBS, were added to the wells. The final concentration of dimethyl sulfoxide used for MTT solubilization was 0.2% (v/v). Following incubation for 48 h, 100 μl medium was removed from each well and 20 μl MTT solution (5 mg/ml PBS) was added. Following incubation for 4 h, the optical density was evaluated at 570 nm. Growth inhibition rates were calculated as a percentage of the parallel negative controls. Each experiment was performed three times.

Cell Matrigel invasion assays. Tumor cell invasion through a reconstituted basement membrane (Matrigel; Sigma-Adrich, Carlsbad, CA, USA) was assayed as previously described (20). In 24-well Transwell cell culture chambers (Chemicon, Temecula, CA, USA), polycarbonate filters (pore size, 8 μm) were precoated with 1 μg fibronectin on the lower surface, following which 5 μg/10 μl Matrigel was applied to the upper surface of the filters. Uncoated wells were used as a negative control. The filters were first dried and washed in PBS, and following rehydration, the SMMC-7721 and CSQT-1 (5×10^5) cells were suspended in RPMI-1640 containing 0.1% bovine serum albumin. The filters were then pretreated with the tested samples for 30 min on ice, added to the upper chamber, and then incubated at 37°C for 12 h. Following incubation, the cells that had invaded the lower chamber and attached to the lower surface of the filter were stained with calcein and quantified at excitation and emission wavelengths of 495 and 515 nm, respectively. Four high-power fields were analyzed for each well.

Quantitative polymerase chain reaction (qPCR). Cells were collected at specified times and total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), in accordance with the manufacturer's instructions. cDNA was constructed as a template to perform qPCR with COX-2, VEGF and β-actin specific primers. The qPCR analyses for VEGF and COX-2 transcription were performed in single microcapillary tubes using a LightCycler™ (Roche Diagnostics, Basel, Switzerland) and SYBR® Green Tag ReadyMix™ (Sigma-Aldrich). Cycling parameters were optimized as follows: Denaturation at 94°C (10 sec), annealing at 55°C (5 sec), extension at 72°C (24 sec) and detection at 80°C (1 sec). Each microcapillary tube contained 7.1 μl nuclease-free H_2O, 10 μl SYBR reagent, 0.5 μl template cDNA, 1.6 μl MgCl$_2$ (25 mM) and 0.8 μl primer mixture (25 pmol/μl). Cycler software was used to quantify COX-2 and VEGF mRNA expression levels.

Cell cycle analysis using flow cytometery. Treated (40 μg/ml cidan for 24 h) and untreated CSQT-1 cells were harvested, washed with PBS and suspended (10^6/ml) in 1.5 ml hypotonic fluorochromic solution [50 mg/ml propidium iodide (PI) in 0.1% sodium citrate plus 0.1% Triton X-100; Sigma-Aldrich] for 60 min at 48°C in the dark. PI fluorescence was analyzed using a FACScaliber flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) with ModFIT cell cycle analysis software version 2.01.2 (BD Biosciences). The experiments were repeated three times for each condition.

Statistical analysis. Data were analyzed using the unpaired t-test with two-tailed P-values or analysis of variance (for multiple comparisons) to calculate the statistical significance between the control and treatment groups. Levels of total bilirubin, direct bilirubin and albumin, the ratio of albumin to globin, prothrombin time, percentage of globin in protein electrophoresis and α-L-fucosidase were compared between the groups using analysis of variance. The Wilcoxon rank sum test was used for comparisons of AFP, carcinoembryonic antigen, carbohydrate antigen 19-9, alanine aminotransferase, aspartate aminotransferase, ALP and hepatitis B virus-DNA/1,000 between the groups. Additional categorical variables were compared between the groups using Fisher's exact test. The results are presented as the mean ±standard error of the mean, and P<0.05 was considered to indicate a statistically significant difference.

Results

No significant variation in the basic characteristics of the cidan and control patients. Although statistically significant differences [AFP, hepatitis B e antigen positive, anti-hepatitis C virus positive and portal clamping duration] were observed in the basic characteristics between the two groups prior to surgery, the majority of the parameters did not significantly vary (Table I). The pathological data at day 2 following surgery indicated that there were no statistically significant differences between the cidan treatment and control groups, despite a reduction in the occurrence of incomplete tumor capsules in the cidan treatment group (Table II).

Differences in the OS and DFS times between the cidan and control groups. The two-year OS rate of the patients that received postoperative cidan as adjuvant therapy was 77%, while the OS rate of the solely hepatoprotective drug treatment group was 58% (Table III; P=0.0031). The two-year DFS rate in the cidan treatment group was 36%, while in the control group, the DFS was 24% (Table IV; P=0.006). A Cox regression model was used to analyze the effect of postoperative adjunctive cidan medication on the OS and DFS rates of patients with HCC following surgery (Figs. 1 and 2), which demonstrated that the use of adjuvant cidan therapy reduced tumor recurrence and improved the survival times.

Anticancer effect of cidan in the Hepa1-6 implanted male C57BL/6 mouse model. In order to further analyze the effect of cidan on hepatic tumor cells, a mouse HCC model was used. Following culturing, 2×10^6 mouse Hepa1-6 hepatoma cells were injected into the subcutaneous tissue of C57BL/6 mice. After seven days, the mice were treated with high (4.80 mg/kg) or low (1.92 mg/kg) doses of cidan, or with distilled water as a control. The tumors were removed from the mice after one, two or three weeks following the start of treatment. The results revealed that the average weight of the tumors after two weeks of high-dose cidan treatment was 50% lower when compared with the control group. Low-dose cidan treatment reduced the tumor size to a lesser extent when compared with the high-concentration cidan treatment. The same trend was observed after three weeks of treatment (Table V).

Table I. Preoperative comparison of patient characteristics between the two treatment groups.

Variable	Control group (n=280)	Treatment group (n=92)	P-value
Age, years	49.16±10.824	50.64±10.553	0.252
Male, n (%)	226 (80.7)	84 (91.3)	0.018
Female, n (%)	54 (19.3)	8 (8.7)	
AFP, μg/l	559.413±554.8254	367.926±501.8287	0.002
AFU, U/l	33.614±1.0493	33.391±0.9514	0.866
ALP, U/l	114.964±101.4081	107.315±58.3713	0.493
ALT, U/l	50.748±50.7809	58.627±122.0881	0.382
AST, U/l	54.581±46.0912	64.865±140.9475	0.289
CA19-9, U/ml	38.078±84.1362	25.833±22.6981	0.169
CEA, μg/l	3.201±6.7797	3.242±3.7395	0.956
GGT, U/l	116.793±120.5559	123.686±126.3800	0.639
Albumin, g/l	41.173±3.9815	41.974±4.1169	0.098
Ratio of albumin and globin	1.341±0.2238	1.384±0.2637	0.131
Size of tumor, cm	9.1350±4.74699	9.2489±4.91545	0.843
Prealbumin, mg/l	220.018±57.9002	228.902±56.9922	0.201
Total bilirubin, μmol/l	15.426±6.4219	14.754±5.4803	0.368
Direct bilirubin, μmol/l	5.730±3.0187	5.351±2.1050	0.265
Prothrombin time, sec	12.202±.9329	12.065±.9718	0.228
Hepatic portal blocking time, min	16.98±11.048	13.60±7.507	0.001
Intraoperative bleeding, ml	463.11±616.957	373.37±412.807	0.115
Intraoperative blood transfusion, ml	358.07±1479.115	158.70±460.328	0.204
Globin in protein electrophoresis, %	20.3164±4.29137	20.1241±4.66117	0.715
Anti-HBcAg, n (%)			
Positive	269 (96.1)	91 (98.9)	0.308
Negative	11 (3.9)	1 (1.1)	
Anti-HBeAg, n (%)			
Positive	175 (62.5)	62 (67.4)	0.581
Negative	105 (37.5)	30 (32.6)	
HBeAg, n (%)			
Positive	108 (38.6)	24 (26.1)	0.030
Negative	172 (61.4)	68 (73.9)	
Anti-HCV, n (%)			
Positive	4 (1.4)	6 (6.5)	0.017
Negative	276 (98.6)	86 (93.5)	
Microscopic vascular invasion, n (%)			
Yes	53 (18.9)	15 (16.3)	0.572
No	227 (81.1)	77 (83.7)	
Portal vein tumor thrombus, n (%)			
Yes	38 (13.6)	15 (16.3)	0.515
No	242 (86.4)	77 (83.7)	
Satellite nodules, n (%)			
Yes	70 (25.0)	16 (17.4)	0.133
No	210 (75.0)	76 (82.6)	
Tumor size, n (%)			
≤5 cm	69 (24.6)	25 (27.2)	0.628
>5 cm	211 (75.4)	67 (72.8)	
Tumor size, n (%)			
≤10 cm	176 (62.9)	54 (58.7)	0.476
>10 cm	104 (37.1)	38 (41.3)	
Tumor number, n (%)			
Single tumor	231 (82.5)	83 (90.2)	0.077
Multiple tumors	49 (17.5)	9 (9.8)	

Table I. Continued.

Variable	Control group (n=280)	Treatment group (n=92)	P-value
Rupture of tumor, n (%)			
Yes	5 (1.8)	3 (3.3)	0.414
No	275 (98.2)	89 (96.7)	

AFP, α-1-fetoprotein; AFU, α-L-fucosidase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; GGT, γ-glutamyl transferase; HBcAg, hepatitis B c antigen; HBeAg, hepatitis B e antigen; HCV, hepatitis C virus.

Figure 1. Comparison of the postoperative OS rates of patients with HCC treated with liver-protection drugs only (control group) or with liver-protection drugs and cidan (treatment group). HCC, hepatocellular carcinoma; OS, overall survival.
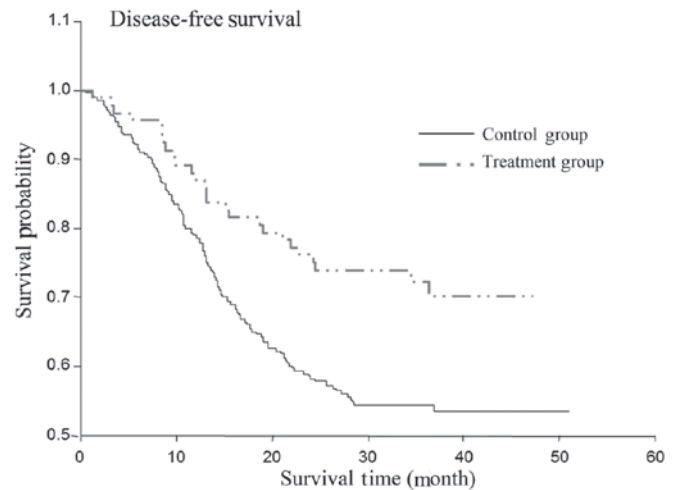

Figure 2. Comparison of the postoperative DFS times of patients with HCC treated with liver-protection drugs only (control group) or with liver-protection drugs and cidan (treatment group). HCC, hepatocellular carcinoma; DFS, disease-free survival.

Cidan inhibits COX-2 and VEGF expression, is toxic in the long-term and inhibits the cell invasion capacities of hepatic tumor cells. SMMC-7721 tumor cells were cultured and treated with cidan, following which the transcription levels of COX-2 and VEGF were analyzed and changes in the tumor cell growth rates were determined. As shown in Fig. 3, the mRNA expression levels of COX-2 and VEGF in the cells treated with cidan were significantly decreased compared with the control. MTT cytotoxicity assays were then performed to determine the effect of cidan on the viability of SMMC-7721 and CSQT-1 cell lines. Cidan exhibited statistically significant cytotoxic effects on SMMC-7721 cells after four and five days (P<0.01), and on CSQT-1 cells after five days (P<0.01), when applied at a dosage of 40 µg/ml (Fig. 4). In addition, Matrigel cell invasion capacity analysis using a Transwell chamber assay revealed that cidan (40 µg/ml) significantly suppressed SMMC-7721 (P<0.01) and CSTQ (P<0.001) cell invasion through Matrigel-coated filters (Fig. 5).

Cidan induces pronounced G2/M cell cycle arrest in CSQT-1 cells. As shown in Fig. 6, 40 µg/ml cidan application for 24 h increased the number of cells in the G1 and G2/M cell cycle phases. In addition, the number of cells in the S cell cycle phase was reduced, indicating that the hepatic cancer cell proliferation rate was reduced.

Therefore, the results revealed that cidan positively affected the DFS and OS rates of patients with HCC. Cidan also reduced mouse model hepatic tumor cell growth in a dose-dependent manner, reduced COX-2 and VEGF expression levels and exhibited cytotoxic effects in hepatic cancer cells. In addition, cidan induced G2/M and G1 cell cycle arrests *in vitro*.

Discussion

In the present study, cidan was demonstrated to improve the postoperative DFS and OS rates in patients with HCC (Figs. 1 and 2), as well as reduce the growth of subcutaneously implanted HCC tumors in mouse models in a dose-dependent manner (Table V). Cidan controls the progression of HCC effectively via a number of oxidative stress and inflammatory reactions, where several associated cytokines and signaling pathways have been confirmed to affect the pathogenesis of HCC (21,22). A number of phytochemicals exhibit anti-inflammatory effects, such as curcumin, a polyphenolic compound derived from rhizomes of *Curcuma*. Curcumin mediates the suppression of nuclear factor-κB (NF-κB), the master switch in the inflammatory cascade (23). NF-κB activation is known to regulate several key inflammatory mediators, including cytokines, chemo-

Table II. Comparison of postoperative pathological determinations between the two groups of patients.

Variable	Liver protection group	Cidan group	P-value
Cell type, n (%)			
Coarse trabecular	190 (67.9)	54 (58.7)	
Coarse trabecular-pseudo ductular	2 (0.7)	1 (1.1)	
Coarse trabecular-hyaline	3 (1.1)	-	
Super fat	1 (0.4)	-	
Mixed	2 (0.7)	-	
Pseudo ductular	4 (1.4)	8 (8.7)	
Others	2 (0.7)	-	
Hyaline	8 (2.9)	2 (2.2)	
In groups and sheets	8 (2.9)	10 (10.9)	
In groups and sheets-hyaline	-	1 (1.1)	
Fine trabecular	56 (20.0)	16 (17.4)	
Fine trabecular-super fat	1 (0.4)	-	
Fine trabecular-pseudo ductular	3 (1.1)	-	
Tumor capsule, n (%)			
Total	280 (100.0)	92 (100.0)	
Incomplete	89 (31.8)	19 (20.7)	0.0119
Complete	95 (33.9)	47 (51.1)	0.0598
Without	96 (34.3)	26 (28.3)	0.4658
Satellite nodules, n (%)			
Total	280 (100.0)	92 (100.0)	
Without	210 (75.0)	76 (82.6)	0.1547
With	70 (25.0)	16 (17.4)	0.2609
Microscopic vascular invasion, n (%)			
Total	280 (100.0)	92 (100.0)	
Without	227 (81.1)	77 (83.7)	0.6427
With	53 (18.9)	15 (16.3)	0.7587
Cell grading, n (%)			
Total	280 (100.0)	92 (100.0)	
I	1 (0.4)	1 (1.1)	0.5843
II	63 (22.5)	22 (23.9)	0.8896
III	214(76.4)	68 (73.9)	0.9269
IV	2 (0.7)	1 (1.1)	0.5759

P-value, Liver protection vs.Cidan group.

Table III. Effect of antitumor medication (cidan) on the prognosis of patients (OS rate).

Subgroup	HR	95% CI lower	95% CI upper	P-value
Cidan (+) vs. (-)	0.476	0.292	0.778	0.0031

Results were determined using a Cox regression model. HR, hazard ratio; CI, confidence interval; OS, overall survival.

Table IV. Effect of antitumor medication (cidan) on the prognosis of patients (DFS time).

Subgroup	HR	95% CI lower	95% CI upper	P-value
Cidan (+) vs. (-)	0.646	0.473	0.882	0.0060

Results were determined using a Cox regression model. HR, hazard ratio; CI, confidence interval; DFS, disease-free survival.

Table V. Inhibition of Hepa1-6 cell xenograft tumor growth in mice by cidan treatment.

Treatment	Cidan dose, mg/kg	Animals, n	Animal weight, g	Tumor weight, g	Inhibition ratio, %
1 week					
Control	-	10	28.8±3.5	2.0±0.7	-
Cidan low	1.92	10	28.7±2.3	1.8±0.7	9.0
Cidan high	4.80	10	28.2±4.2	1.6±0.5[a]	22.0
2 weeks					
Control	-	10	29.0±3.1	2.2±0.8	-
Cidan low	1.92	10	27.4±2.5	2.1±0.7	15.4
Cidan high	4.80	10	27.8±4.3	1.1±0.3[b]	30.2
3 weeks					
Control	-	10	28.2±3.0	3.2±0.9	-
Cidan low	1.92	10	28.4±2.3	1.8±0.5	18.0
Cidan high	4.80	10	27.8±4.2	1.1±0.4[b]	35.0

[a]$P<0.05$ and [b]$P<0.01$, vs. control.

Figure 3. COX-2 and VEGF mRNA expression levels in SMMC-7721 cells following treatment with cidan. [***]$P<0.001$ and [*]$P<0.05$, vs. control group (saline). COX-2, cyclooxygenase-2; VEGF, vascular endothelial growth factor.
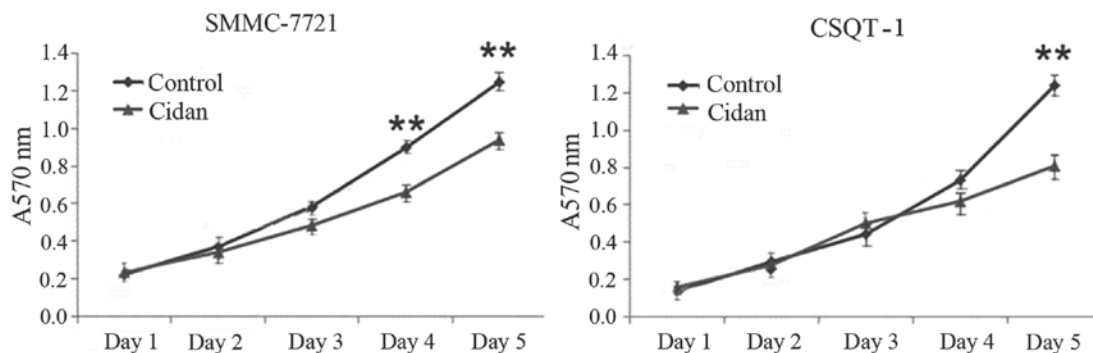

Figure 4. *In vitro* cytotoxic activity of cidan in human tumor cell lines. [**]$P<0.01$, vs. Cidan group (saline).

kines and kinases, which have been shown to play critical roles in the pathogenesis of the majority of chronic illnesses (24) The curcumin-mediated attenuation of the NF-κB-activated

inflammatory cascade is a critical mechanism of its therapeutic effects (25). Although cidan is the most widely known and effective curcuminoid present in China, this drug also
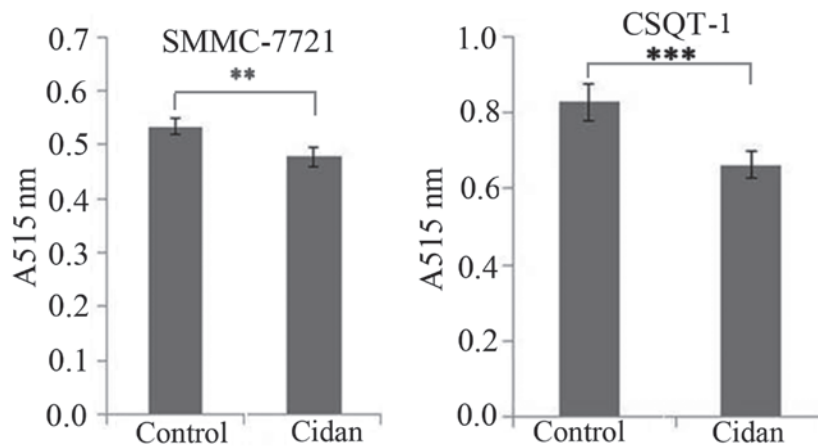
Figure 5. Inhibition of Matrigel cell invasion. SMMC-7721 and CSQT-1 cells were incubated with cidan-containing medium for 12 h. **P<0.01 and ***P<0.001, cidan treatment group vs. control group (saline).
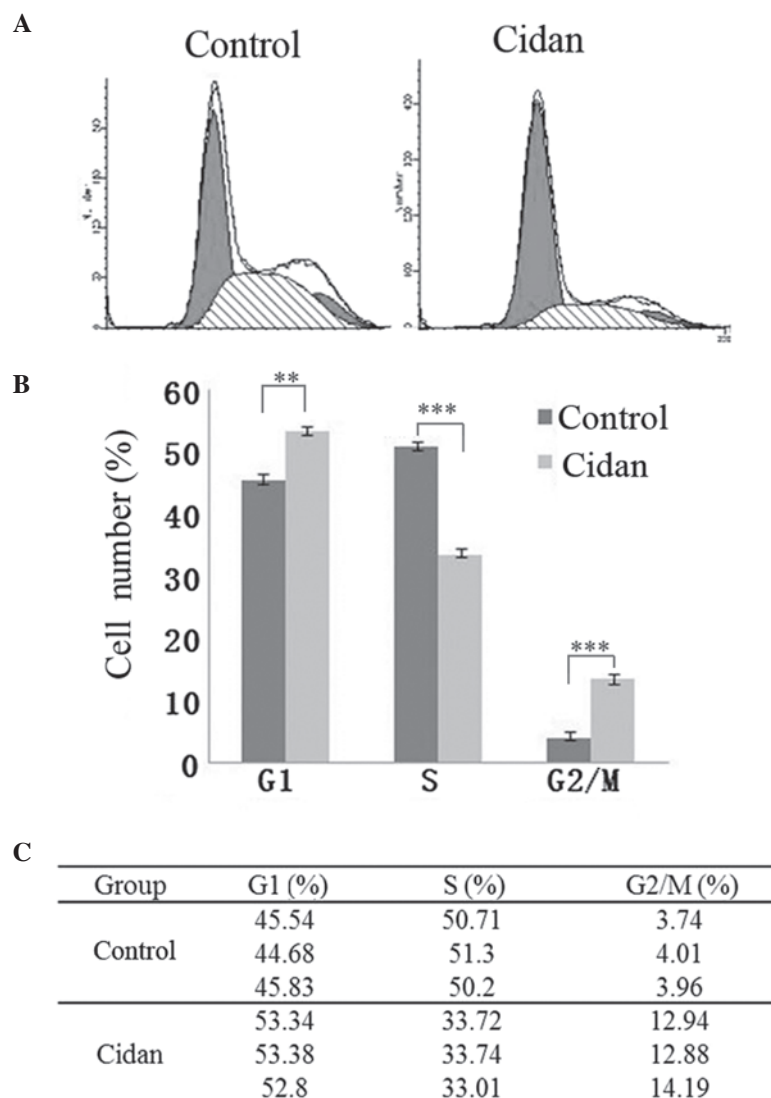

Group	G1 (%)	S (%)	G2/M (%)
Control	45.54	50.71	3.74
	44.68	51.3	4.01
	45.83	50.2	3.96
Cidan	53.34	33.72	12.94
	53.38	33.74	12.88
	52.8	33.01	14.19

Figure 6. Flow cytometric analysis of CSQT-1 cells following 24 h incubation with 40 μg/ml cidan or saline (control). (A) Representative flow cytometry images; (B) percentage chart and (C) numerical analysis of the cells in the various cell cycle stages. **P<0.01 and ***P<0.001, vs. control cells (saline treatment).

contains more than three additional polyphenolic curcuminoids. Elemene is the anticancer component extracted from Rhizoma Curcumae, which has been demonstrated to have an anticancer effect by inducing apoptosis in tumor cells (26,27). Recently, an additional study demonstrated that curcumin induced G2/M cell cycle arrest via targeting the

anaphase-promoting complex/cyclosome protein, Cdc27, and inducing enhanced apoptosis rates (28). The results of the present study were in accordance with these observations, and marked G2/M phase cell cycle arrest was observed in the CSQT-1 cells following incubation with cidan (Fig. 6). In addition to the enhanced G2/M cell cycle arrest of CSQT-1 cells, cidan was shown to inhibit COX-2 and VEGF expression levels and prevent hepatic cancer cell invasion activity (Figs. 3 and 5). These observations indicated that there was a synergistic cell growth inhibition of SMMC-7721 and CSQT-1 cells, accompanied with an increase in apoptosis rates and a decrease in NF-κB activation with concomitant lowering of COX-2 expression levels, as demonstrated in a previous study (29). Tanshinone, the effective component extracted from *Salvia miltiorrhiza*, is also found in cidan and has known anticancer properties. This compound has been demonstrated to dissolve fibrin wrapped on the surface of cancer cells, increase the immunogenicity of tumors, induce cell differentiation and gradually reduce the malignancy of cancer cells (30). Furthermore, cidan capsules contain *Brucea javanica* oil, extracted from *Brucea javanica*, which has been shown to induce apoptosis and decrease cell proliferation; thus, demonstrating favorable antitumor activity (31). Additional ingredients include Cirrhopetalanthrin, a type of chemical composition extracted from *Cremastrae appendiculata*, which exhibits anticancer activity by inhibiting mitosis, and Astragalus, which promotes the body to monitor tumor cells and produce interferon, increasing lymphokine-activated killer and natural killer cell activity and strengthening the phagocytic function of the reticuloendothelial system to kill cancer cells (32). Furthermore, a low therapeutic cidan concentration in patients has been cited as a reason for the lack of sufficient success in clinical trials. In the present *in vivo* study, this hypothesis was supported since tumor growth was shown to diminish in the mouse model in a dose-dependent manner (Table V).

In conclusion, the present study demonstrated that cidan effectively inhibited proliferation and was toxic to hepatoma cells. Furthermore, COX-2 and VEGF expression levels were found to be downregulated following cidan administration. Thus, cidan therapy may effectively reduce tumor recurrence and improve the OS time when applied as postoperative treatment for HCC. However, further studies are required to clarify the exact mechanisms involved in the antitumor effects of cidan, with particular focus on the activity of the individual ingredients.

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