

Original Article

The mechanism of Dedu seven-ingredient pill with safflower in resisting liver cancer

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Abstract: Objective: The goal of this study was to investigate the role of Dedu seven-ingredient pill with safflower in inhibiting proliferation of human HepG2 liver cancer cells *in vitro* and in mice and verify the targeted regulation relationship between miR-23a and DAPK1. Method: The Dedu seven-ingredient pill with safflower was prepared to treat the human hepatocellular carcinoma cell strain HepG2. Then, the MTT, scratch wound and cell invasion experiments were performed. The N-nitroso compound (DEN) was used to establish the mice model with liver cancer. Expression of the DAPK1 gene mRNA was detected by means of real-time quantification PCR. The expression of the DAPK1 protein was detected with the Western blot method. The targeted regulation relationship between miR-23a and DAPK1 was further verified by a dual-luciferase reporter gene experiment. Result: Dedu seven-ingredient pill with safflower played a significant role in decreasing the invasion and migration capacity of the HepG2 liver carcinoma cells. The fluorescent real-time quantification PCR and the Western Blotting result indicated that the mRNA expressed by the DAPK1 gene and the expression of the appropriate protein following administration of Dedu seven-ingredient pill with safflower were significantly up-regulated compared with that in the control group ($P < 0.05$). Expression of miR-23a in the Dedu seven-ingredient pill with safflower group was down-regulated by 48% compared with that in the liver cancer model group. The luciferase reporter gene experiment indicated that no significant changes occurred in the activity of the DAPK1-MUT group while the fluorescence intensity of the DAPK1-WT group was decreased significantly ($P < 0.05$). Conclusion: The Dedu seven-ingredient pill with safflower impedes the migration and invasion of the cancer cells and inhibits proliferation of the cancer cells, through down regulating miR-23a level to promote the expression of the DAPK1 protein.

Keywords: Mongolian medicine, Dedu seven-ingredient pill with safflower, liver cancer, miR-23a, DAPK1

Introduction

Primary liver cancer is one of the most common malignant tumors in clinical practice characterized by difficulty in early diagnosis, rapid disease progression and high fatality rate etc. The most recent data indicate that over 600,000 people die of liver cancer each year in the world. Generally, patients with liver cancer have a low 5-year survival rate ranging from 10% to 20% in most countries from 1995 to 2009. The cases of liver cancer in China account for more than 50% of the total number of cases in the world. Approximately 2000,000 to 300,000 people died of liver cancer each year, and the majority of untreated patients died within 1 year after a definite diagnosis was made [3].

The most fundamental characteristic of the traditional Mongolian medicine and pharmacy is holism. The external observation and internal diagnosis are combined to focus on the relationship between viscera, internal organs, appearance, apertures based on the Mongolian medicine culture of “Sangen and Qisu”. In Mongolian medicine, it is believed that the liver cancer is located in the right upper abdomen and below the tabula. The physiological functions of liver cancer are to divide the heat energy [4]. Therefore, the hepatic diseases largely belong to febrile symptoms and the cool-natured medicines are primarily used in treatment.

The Mongolian patent medicine, Dedu seven-ingredient pill with safflower (Dedu Gurigumu-7),



Figure 1. Composition of Dedu seven-ingredient pill with safflower.

was recorded as *Tongwagejide* in the 18th century [5] and currently entitled in *Fascicule for Mongolian Medicine, Drug Standard of the Ministry of Health of the People's Republic of China*. It is a common formula for treatment of liver blood stasis and blood heat. The indication aims to heat from blood. The drug is therefore used to treat liver blood stasis and blood heat, hepatalgia, yellow skin, and yellow urine. The formula mainly comprises safflower in combination with gypsum fibrosum, ephedra, Akebia quinata (Houtt.) Decne, scabiosa, viola philippica, and Terminalia chebula Retz (**Figure 1**).

Accumulative evidence shows that miRNAs play an important role in occurrence and progression of tumors since 2002 when the Croce research group first reported the abnormality of miRNA was associated with tumor [6]. The occurrence and progression of liver cancer is multi-factorial and complex. The role of miRNAs in liver cancer is also frequently studied. Currently, it is reported that miR-21, miR-17-92 cluster, miR-23a-24 cluster, miR-101 cluster, miR-106b-2 cluster, miR-122, miR-221, miR-222, miR-223, miR-224 etc. are associated with the occurrence of liver cancer. These findings indicate that miRNA plays a vital role in

occurrence and progression of liver cancer [7-9].

In this study, the function of Dedu seven-ingredient pill with safflower and the potential mechanism was explored in liver cancer.

Material and method

Material

Dedu seven-ingredient pill with safflower (Ulanhot, China-Mongolia Pharmaceuticals Co., Ltd., Production Batch No.: 150801, Approval Number: z15021590) was ground into powder, dissolved to the desired concentration with double distilled water (5 g original medicine/ml), and filtered with 0.22 μ m filter membrane. The medicine was preserved at 4°C for later use.

The cell strain used in the research was human hepatocellular carcinoma cell strain HepG2 purchased from VCANBIO Cell & Gene Engineering Corp., Ltd. (Tianjin, China). The human kidney epithelial cell line 293T was preserved in the molecular biology experiment center of Inner Mongolia Medical University and cultured in the DMEM culture medium containing 10% FBS at 37°C under 5% CO₂. The cells in the logarithmic growth phase were used for experiment.

C57BL/6 mice of SPF Class weighing 18-22 g (comprising male mice and female mice in equal quantity) purchased from the laboratory animal center of the Inner Mongolia Medical University were used as the experimental animals. Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of our hospital.

Detection of cell proliferation with the MTT method

The cells were divided into the experimental group and the control group. 5×10⁵/mL single-cell suspension was prepared with the cells in each group, and inoculated into a 96-well

microplate with 100 μL per well. 20 μL of MTT (final concentration 5 mg/mL) was added after each well was intervened in darkness for 24, 48, and 72 hours respectively. The cell suspension was incubated in an incubator for 4 hours at 37°C and the supernatant was discarded. 150 μL of DMSO was added. The microplate was shaken on a shaker until the crystals were completely dissolved in DMSO. The A value was detected with a microplate reader (RT-6100, Rayto, Shenzhen, China) at 490 nm. The values of the 5 wells were averaged.

Scratch-wound experiment

The blank control group was subject to no treatment. In the Dedu seven-ingredient pill with safflower group, the cells in each well were scratched and washed with PBS at 24 hours after addition of the solution. The culture medium was changed. The cells were micro amplified 100 folds and photographed at 0, 24, and 4 hours. The differences in scratch wound healing were compared between various experimental groups. The images were processed with the ImageJ 1.48u analysis software. Then, the distance was compared and analyzed.

Cell invasion experiment

The melted Matrigel (BD Company, U.S.) was diluted with DMEM. 100 μL of the diluent was added to upper chamber of the Transwell TM chamber (Corning incorporated, U.S.). The cells were incubated for 4 hours at 37°C until coagulation of the Matrigel. The cells intervened for 48 hours were digested with pancreatin and re-suspended with DMEM containing 10 mL/L FBS. 200 μL of the cells were seeded into the upper chamber as per $1 \times 10^5/\text{mL}$. 600 μL of the DMEM culture medium containing 200 mL/L FBS was added to the lower chamber. The cells were incubated for 24 hours in an incubator containing 50 mL/L CO_2 at 37°C. The cells retained on the surface of the filter membrane were gently removed with cotton swab. The cells were fixed with 40 g/L paraformaldehyde, stained with 1 g/L crystal violet, and washed with PBS. The count of the cells was computed under high power field of the microscope. Five fields of vision were selected for each chamber and the count of cells under each field of vision was recorded. The count of the cells passing through the membrane was computed to indirectly reflect the invasion ability of tumor cells.

Establishment of mouse models with liver cancer induced by N-Nitro compound

C57BL/6 mice aged 4 weeks were placed in a tubular device that could limit their activity and allow them to breathe freely for 25-30 consecutive days as a stress cycle for 6-8 hours each day for a total of 3-4 stress cycles occurring at over an interval of 5-7 days. All mice were randomly divided into the carcinogenesis group and the control group. The mice in the carcinogenesis group were intraperitoneally injected with DEN at a dose of 100 mg/kg, administered the mixture of CCl_4 and olive oil (volume ratio 20:80) at a dose of 0.005 ml/g by gavage twice per week, administered DEN by gavage once at a dose of 50 mg/kg at 3 weeks and given drinking water containing 9% ethanol, and administered CCl_4 at 4 weeks. The dose was increased to 0.008 ml/g. Meanwhile, the mice were fed normal pellet feed. Three mice were randomly sampled from the carcinogenesis group and the control group each at 4, 6, 8, 10, 12, 14, 16, 18, and 20 weeks after cancer induction and subject to laparotomy for observation of the liver surface and color. The liver tissue was excised for subsequent experiments.

Intervention Measures: 36 mice were randomly divided into 3 groups. (1) blank control group; (2) liver cancer model group; (3) Dedu seven-ingredient pill with safflower group (Dedu seven-ingredient pill with safflower administered as per body weight each day (6.5 mg/10 g).

Method of Administration: Dedu seven-ingredient pill with safflower was ground into powder, dissolved to the desired concentration with double distilled water, and filtered with 22 μm screen. Each mouse was intraperitoneally injected with the medicine at a dose of 1 ml and sacrificed by cervical vertebra dislocation at 24 hours after drug withdrawal.

Fluorescent real-time quantification PCR detection of the expression of DAPK1 mRNA

The Small RNAs (<100 nt) from the Dedu seven-ingredient pill with safflower solution were extracted using the Universal Plant MicroRNA Kit (Invitrogen, U.S.). (1) Reverse transcription: cDNA was reversely transcribed with the Taq-Man® MicroRNA Reverse Transcription Kit (ABI Company, U.S.). All operations were completed on ice. The total reaction system was 15 μL : 100 mM dNTPs 0.15 μL , MultiScribe™ Reverse Transcriptase 1.00 μL , 10× Reverse Transcription

Buffer 1.50 μ l, RNase Inhibitor 0.19 μ l, Nuclease-free water 4.16 μ l, hsa-miR-23a or U6 5 $\times$ RT Primer 3 μ l, and RNA sample 5 μ l (10 ng total RNA). The reaction occurred at 16°C, for 30 minutes, at 42°C for 30 minutes, and at 85°C for 5 minutes. The cDNA synthesized by reverse transcription was preserved at -20°C for later use. (2) Real-time PCR: A relatively quantitative analysis was conducted with U6 as the internal reference. The PCR reaction occurred in a real-time quantification in a PCR PCRA amplifier (7500, ABI, U.S.). The reaction of each sample was replicate in two wells. The reaction system comprised cDNA 1.33 μ l, hsa-miR-23a or U6 20 $\times$ Real Time primer 1 μ l, TaqMan 2 $\times$ Universal PCR Master Mix 10 μ l, Nuclease free water 7.67 μ l. The total reaction system was 20 μ l. The reaction occurred at 95°C for 10 minutes, at 95°C for 15 seconds, at 60°C for 1 minute. There were a total of 40 cycles [11].

Detection of the expression of the DAPK1 protein with Western blot

The experimental cells were transferred to a centrifuge tube. The protein was extracted and quantified according to the operating steps in the instructions for the kit. The Western blot-related reagents were purchased from U.S. Sigma. A total of 30 μ g of the protein was subject to electrophoresis with 10% SDS-polyacrylamide gel. The gel containing target protein following electrophoresis was removed according to the Marker protein separation band of the molecular weight of the target protein. It was transferred to the PVD membrane, blocked with 5% skim milk powder for 2 hours at room temperature. The blocked PVDF membrane was immersed in the primary antibody solution properly diluted with TTBS and shaken gently overnight. The membrane was washed at room temperature and immersed in the second antibody solution marked with horse radish peroxidase and diluted with TTBS as per 1:10000 for reaction for 2 hours. The ECL chemiluminescence technique was used. The imaging analysis software was used for relatively quantitative analysis of the signal intensity of Western blot development band. The scanning gray value was expressed with the optical density value.

Plasmid transfection and luciferase reporter gene experiment

miR-23a had 10 sequences fully consistent with the WT-DAPK1-3'UTR terminal while the

design of MUT-DAPK1-3'UTR completely disrupted the 10 base sequences. The Lipofectamine 2000 liposome-mediated transfection method was used to co-transfect the luciferase reporter vector pGL3M-DAPK1-3'UTR (50 ng) and the control plasmid pcDNA3.1 (10 ng) (GeneCopoeia Company, U.S.) into the 293T cells and named the experimental group pGL3M-WT-DAPK1-3'UTR, positive control group pGL3M-MUT-DAPK1-3'UTR, and negative control group pGL3M. The result was detected with dual-luciferase and expressed with the ratio of the firefly luciferase activity to the sea pansy luciferase activity [12].

Statistical analysis

The miRNA chip expression profile was subject to an unsupervised clustering analysis (TMeV version 4.0) with SAM and TIGR Mutiple Array Viewer software package (TMeV version 4.0). The fluorescent real-time quantification PCR used the software Sequence Detection system (SDS) 2.3 for data analysis. Expression level of miRNA was expressed with the Δ Ct value (Ct miRNA-Ct U6). The biological experimental data are expressed with mean $\pm$ standard deviation (mean $\pm$ SD). The differences between the two groups were subject to a Student's t test. The inter-group data were compared with the Chi-square test or the Fisher exact probability method. The differences were statistically significant when $P < 0.05$. The statistical analysis used the software SPSS 18.0.

Result

Effect of Dedu seven-ingredient pill with safflower on the proliferation capacity of the HepG2 cells

The MTT experimental result indicated that the OD value in the blank group decreased to 0.44 from 0.93 in terms of cell proliferation rate at 72 hours after administration with Dedu seven-ingredient pill with safflower ($P < 0.01$, **Figure 2**).

Effect of the Dedu seven-ingredient pill with safflower on the migration and invasion capacity of the HepG2 cells

The Transwell TM experimental result showed that the invasion capacity of the HepG2 hepatocellular carcinoma cell strain decreased significantly at 48 hours after the cells were intervened with Dedu seven-ingredient pill with safflower compared with that in the control group. The amount of the cells passing through the

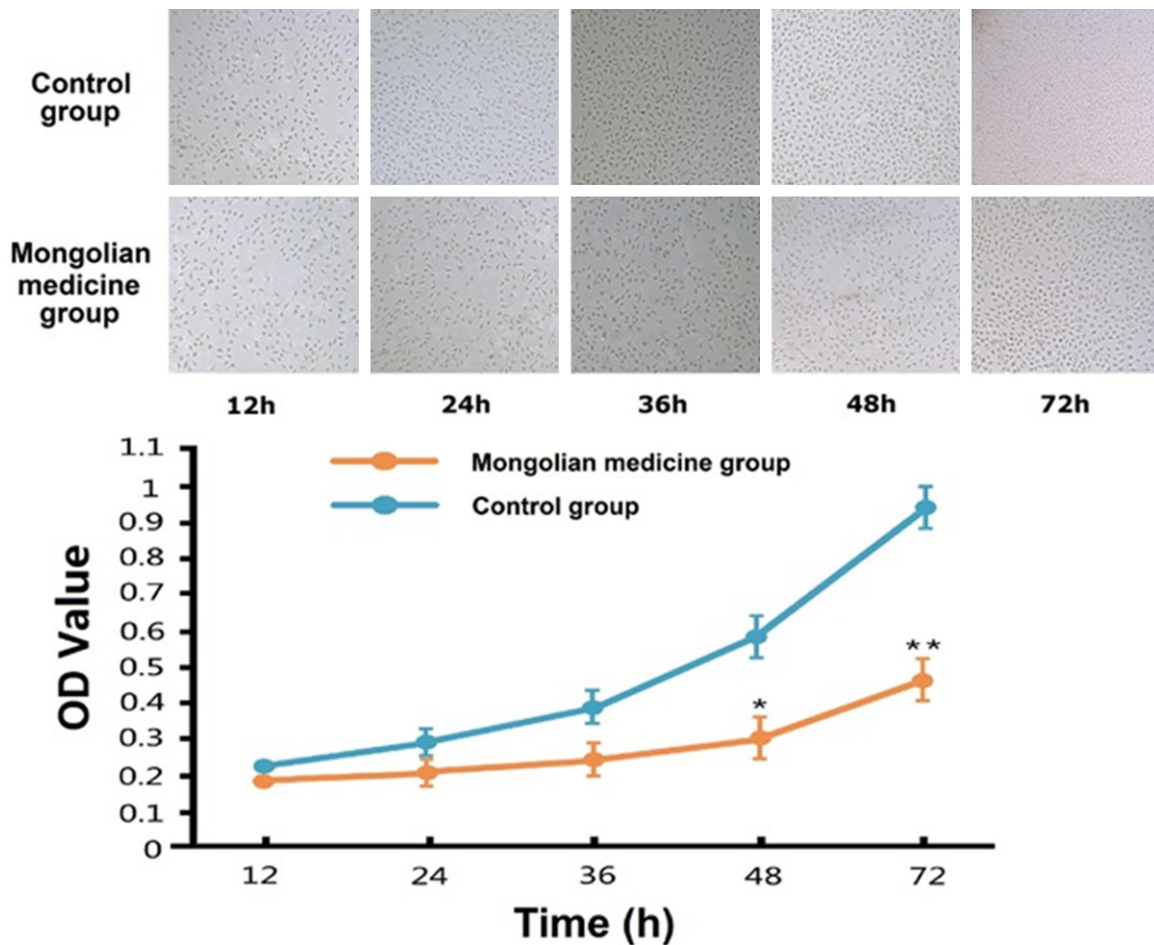


Figure 2. Effect of Dedu seven-ingredient pill with safflower on the proliferation capacity of liver cancer HepG2 cells. The upper figure presents the states of the cells in the hepatocellular carcinoma cell group and the Mongolian medicine group at 12-72 hours. The lower figure shows the OD value at each time point. There were significant differences between the Mongolian medicine group and the control group at 48 hours. The OD value decreased significantly at 72 hours. *indicates $P < 0.05$, **indicates $P < 0.01$. (200 $\times$ magnification).

basilar membrane was decreased. The Dedu seven-ingredient pill with safflower may play a significant role in decreasing the invasion capacity of the liver cancer HepG2 ($P < 0.05$, **Figure 3A**). In the scratch experiment, migration occurred in the blank group at 24 hours and the scratch in the blank group shortened significantly at 48 hours compared with that in the experimental group (**Figure 3B**), indicating that the Dedu seven-ingredient pill with safflower contributed to the decrease of migration capacity of the liver HepG2 liver carcinoma cells.

Roles of Dedu seven-ingredient pill with safflower in down-regulating the level of miR-23a and the expression of DAPK1 in liver cancer

The level of miR-23a in the model rats with liver cancer exhibited a significant rising trend and

increased from -2.9 folds to 2.4 folds compared with that of the mice in the control group (**Figure 4A**). The level of miR-23a of the model mice with liver cancer increased by 2.4 times after addition of the vacant plasmid but significantly decreased by approximately 1.2 times after administration of the Mongolian medicine ($P < 0.05$). Only the level of miR-23a in the Mongolian medicine group decreased significantly after miR-23a mimics were administered to the blank control group, liver cancer model group, and the Mongolian medicine group ($P < 0.05$) (**Figure 4B**). The real-time quantification fluorescent PCR method was used to detect the relative expression of mRNA of the DAPK1 gene. The relative fluorescence value result indicated that the relative expression value was 1.32 ± 0.25 at 72 hours in the hepatocellular carcinoma cell group and 2.15 ± 0.16 in the Mongolian medicine group (**Figure 4C**).

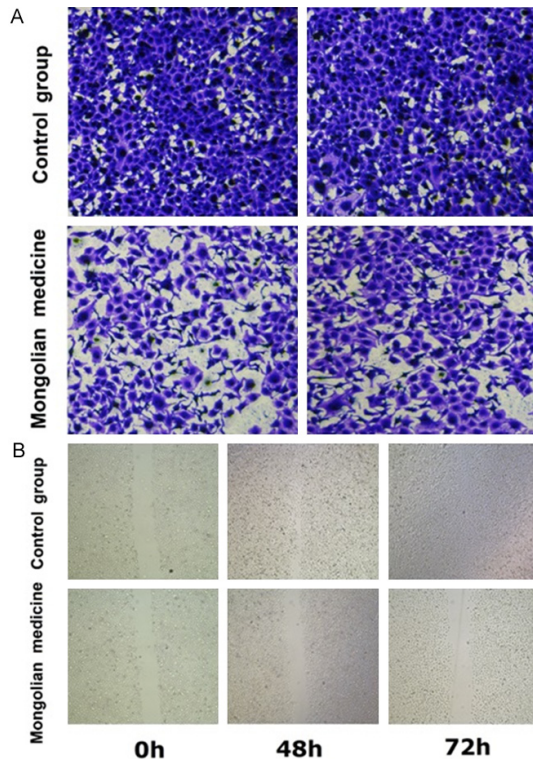


Figure 3. Invasion potential of HepG2 liver carcinoma cells after Mongolian medicine treatment was analyzed using Transwell invasion assay (400× magnification). The migration capacity of HepG2 liver carcinoma cells after Mongolian medicine treatment was analyzed using a wound healing test (200× magnification).

The relative expression value was 1.96 ± 0.22 at 72 hours in the group with miR-23a inhibitor administered to mice, which was significantly higher than that of 1.48 ± 0.20 in the vacant plasmid group ($P < 0.05$) (**Figure 4D**).

Role of miR-23a mimics in decreasing the protein expression level of DAPK1

The qPCR result indicated that the miR-23a mimics significantly decreased the level of DAPK1 mRNA. Additionally, the Western blot experimental data showed that the protein expression level of DAPK1 could be decreased at 72 hours after the HepG2 cells were over expressed with miR-23a (**Figure 5A, 5B**).

Verification of the target gene DAPK1 of miR-23a by means of luciferase report

Three types of on-line software, TargetScan, PicTar, and miRanda were used to predict the bioinformatics of the miRNA target gene and

screen for many tumor suppressor genes associated with proliferation, migration, and progression. The result suggests that there may be a target regulation relationship between miR-23a and the DAPK1 gene. Based on such prediction, WT-DAPK1 and MUT-DAPK1 (**Figure 5C**) were established. The luciferase report result of the 293T cells showed that there were no significant changes in pGL3M-MUT-DAPK1-3'UTR and pGL3M-WT-DAPK1-3'UTR in the control group relative to the pGL3M in the vacant plasmid group. No significant changes occurred in the activity in the MUT group, however the fluorescence intensity in the WT group decreased significantly after addition of miR-23a mimics, suggesting that miR-23a targeted the DAPK1 by specific sequence binding (**Figure 5D**).

Discussion

The Mongolian medicine has a history of about 1200 years. The cold climate of China's northern grassland leads to long-term excessive alcohol consumption among peoples. It frequently causes pathological changes in liver and a range of diseases such as liver cancer etc. It is generally believed that berberis sargentiana can effectively treat such diseases [13, 14]. However, there has been no relative research on the role of Dedu seven-ingredient pill with safflower in resisting liver cancer. As liver cancer is treated on the basis of the Mongolian medicine theory, the paper involves the Mongolian medicine but does not validate the exact function of its active ingredients, which conform to the holistic view and conception of nature in the Mongolian medicine theory [15, 16].

miRNAs are a class of highly-conserved non-coding small molecular RNAs that widely participate in regulation of various living activities such as growth and development, cell apoptosis, proliferation, division etc. [17, 18]. A great deal of research has been conducted on the roles of the protein coding genes in tumorigenesis. However, there is no extensive research on the miR-23a related to liver cancer tumors. Data demonstrate that miR-23a may participate in the occurrence and progression of liver cancer by regulating the cell proliferation, apoptosis, or migration. Down-regulation of the miR-23a can also significantly inhibit proliferation of the hepatocellular carcinoma cells and the *in*

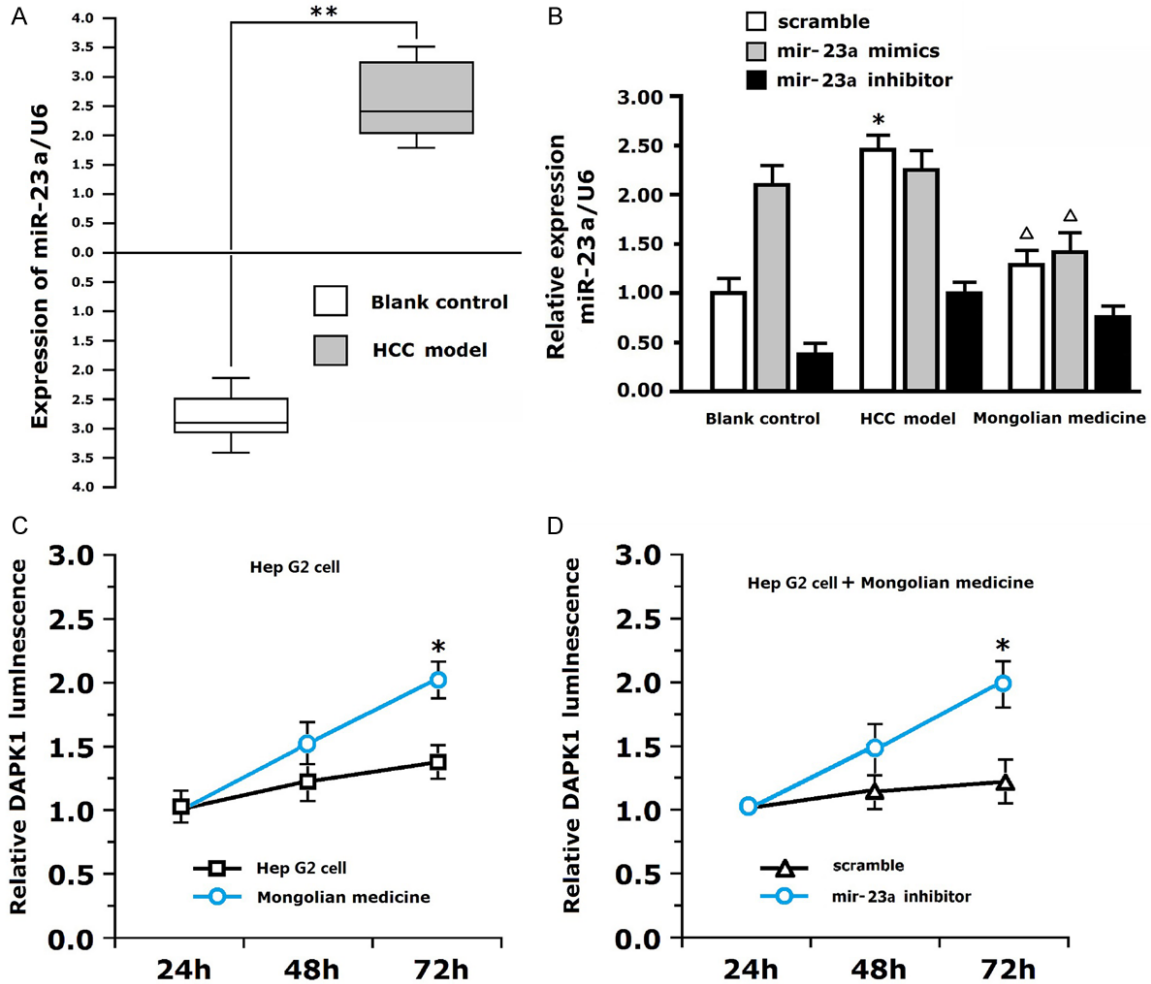


Figure 4. Effect of Dedu seven-ingredient pill with safflower on the invasion and migration capacity of the HepG2 liver carcinoma cells of mice. The upper figure presents the count of the cells passing through the basilar membrane at 48 hours after administration in the Transwell TM experiment (crystal violet staining). The lower figure indicates the cell migration at 48 hours after administration in the HepG2 cell scratch-wound experiment.

vitro migration capacity of the hepatocellular carcinoma cells.

As a tumor suppressor gene participating in cell apoptosis, DAPK1 plays important roles in inducing cell apoptosis, such as cell apoptosis induced by the tumor necrosis factor α , cell apoptosis induced by suicide-related factors, cell apoptosis induced by interferon γ , cell apoptosis induced by the transforming growth factor β , etc. Under normal conditions, DAPK1 can control programmed death of cells and maintain the dynamic equilibrium between cell proliferation and apoptosis. The equilibrium would be disrupted when inactivation occurs. Unlimited cell proliferation would occur and even malignant tumors may also occur. Research finds that abnormal hyper-methylation

occurs in DAPK1 in the Cp G region of the promoter in many types of malignant tumors and cell lines, such as lung cancer, gastric cancer, B cell lymphomas, bladder carcinoma, cervical cancer etc. [20-23]. Previous evidence also unraveled that DAPK1 was also associated with the invasion and metastasis capacity of the tumor cells [24]. In the Lewis carcinoma cell line, restoration of the expression of DAPK1 to the normal level can retard the local growth of tumors and inhibit the pulmonary metastasis capacity of the tumor cells. In mouse lung cancer models, some cell strains with high invasion and metastasis express no DAPK1. Evidence demonstrates that DAPK1 can inhibit carcinogenic transformation induced by c-Myc and E2F [25]. Based on the above research and other research, it is generally accepted that DAPK1 is

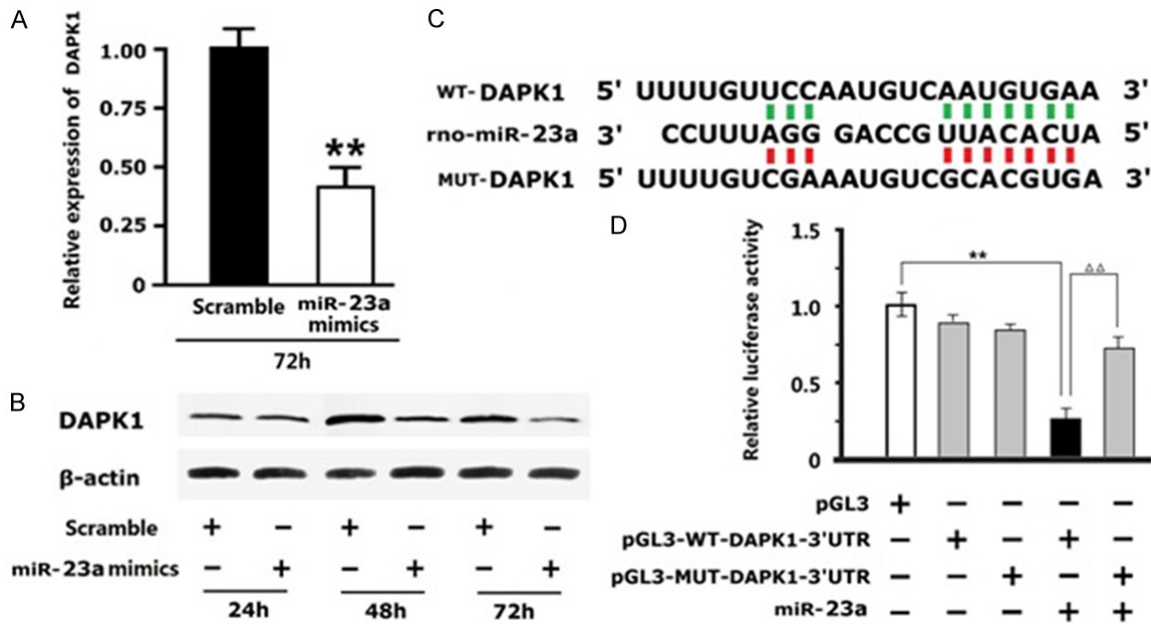


Figure 5. Roles of Dedu seven-ingredient pill with safflower in down-regulating the expression level of miR-23a and up-regulating the expression level of DAPK1. **A:** Expression level of miR-23a in the liver cancer model mice compared with that in the blank control group; **B:** qPCR detection of expression levels of miR-23a in the blank control group, liver cancer model group, and Mongolian medicine group following administration of vacant plasmid, miR-23a mimics, and miR-23a inhibitor. **C:** Expression level of DAPK1 in the liver cancer cells and the cells treated with the Mongolian medicine at 24, 48, and 72 hours after qPCR detection. **D:** qPCR detection of expression level of DAPK1 in the hepatocellular carcinoma cells treated with the Mongolian medicine after addition of the vacant plasmid and miR-23a inhibitor at 24, 48, and 72 hours. *indicates $P < 0.05$ based on a comparison with the control group; **indicates $P < 0.01$ based on a comparison with the control group; Δ indicates $P < 0.05$ based on a comparison with the model group.

a tumor suppressor gene. The Cp G island hyper-methylation in the promoter region leads to gene transcriptional silencing. The gene loses its functions and participates in occurrence and progression of various human tumors. As the methylation state of DNA can be reversed, tumors can be treated by restoring the expression of the tumor suppressor gene that fails to mutate or the lost tumor suppressor gene for recovery of the normal growth and regulation functions of the cells. Based on a prediction with the bioinformatics method, DAPK1 is a potential target gene of miR-23a. The experimental result of the luciferase report has indicated that the luciferase activity in the DAPK1-WT co-transfection group decreases significantly compared with that in the control group. Western-Blot analysis has also demonstrated that the expression level of the DAPK1 protein increases significantly after miR-23a is expressed in the down-regulated hepatocellular carcinoma cells, which demonstrates that DAPK1 is the direct downstream target gene of miR-23a. The cell cycle specificity of miR-23a

can provide a direction for developing medicines similar to methotrexate that blocks the S stage. In-depth research on miRNA will help further explain the molecular mechanism of liver cancer. More miRNAs related to liver cancer are still required to be identified and explored.

Conclusion

In conclusion, these data demonstrate that Dedu seven-ingredient pill with safflower can effectively inhibit migration and proliferation of the tumor cells and promote the expression of DAPK1 by down-regulating miR-23a, which provide a new clue for a deeper understanding for the treatment of liver cancer in the Mongolian medicine.

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Disclosure of conflict of interest

None.

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