Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

Patrinia herba aqueous extract on the proliferation, apoptosis, invasion and migration of hepatocellular carcinoma cells

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Received March 23, 2020; **Accepted** May 17, 2020; **Published** June 5, 2020

Doi: http://dx.doi.org/10.14715/cmb/2020.66.3.18

Abstract: This study aims to study the effect and mechanism of *Patrinia herba* aqueous extract on proliferation, apoptosis, invasion and migration of hepatocellular carcinoma cells. Hepatocellular carcinoma cells MHCC97-H were treated with 2.5, 5, 10 mg/mL *P. herba* aqueous extract, cell counting kit 8 (CCK-8), flow cytometry, plate cloning experiments, and Transwell measured cell survival, apoptosis, colony formation, invasion, and migration, respectively. Real-time quantitative PCR (qPCR) and western blot were used to detect long non-coding RNA (lncRNA) HTR2A-AS1 and expression of proteins P21, Caspase-3, E-cadherin and matrix metalloproteinase-2 (MMP-2), respectively. Transfected pcDNA3.1-HTR2A-AS1 in MHCC97-H cells, or transfected si-HTR2A-AS1 and treat with 10 mg/mL *P. herba* aqueous extract to evaluate their roles in cell proliferation, apoptosis, invasion, and migration. Different concentrations of *P. herba* aqueous extract significantly reduced the survival rate, colony formation, number of migrating cells, number of invading cells, and MMP-2 protein expression of MHCC97-H cells, and obviously increased the cell apoptosis rate, the expression levels of Caspase-3, E-cadherin protein and HTR2A-AS1 (P<0.05), which were all concentration-dependent. Overexpression of HTR2A-AS1 evidently decreased the survival rate, colony formation, number of migrating cells, number of invading cells, and MMP-2 protein levels in MHCC97-H cells, while remarkably enhanced the apoptosis rate of cells, P21, Caspase-3, and E-cadherin protein levels and HTR2A-AS1 expression level (P<0.05). Inhibition of HTR2A-AS1 greatly improved the survival rate, the number of clone formation, the number of migrating cells, the number of invading cells and the expression of MMP-2 protein of MHCC97-H cells treated with *P. herba* aqueous extract, dramatically reducing the cell apoptosis rate, P21, Caspase-3, E-cadherin protein levels and HTR2A-AS1 expression levels (P<0.05). *P. herba* aqueous extract may inhibit the proliferation, invasion and migration of hepatocellular carcinoma cells by up-regulating the expression of HTR2A-AS1 in hepatocellular carcinoma cells and induce apoptosis.

Key words: *Patrinia herba* aqueous extract; Liver cancer; Proliferation; Apoptosis; Invasion; migration; HTR2A-AS1.

Introduction

Liver cancer, as one of the most common malignancies in the world (1), is the third major cause of cancer-related mortality worldwide (2). There are diverse risk factors related to liver cancer, including hepatitis C, hepatitis B, alcohol-related liver disease and non-alcoholic fatty liver disease, obesity and type 2 diabetes (3). So far, many studies have provided oncobiology insights into liver cancer and proposed opportunities for personalized therapy (4), but the detailed mechanism of liver cancer is still lacking. Therefore, it is particularly urgent to investigate liver cancer progress and develop new treatment strategies. *Patrinia herba* first recorded in the "Compendium of Materia Medica" refers to the root or whole plant of *P. scabra* Bunge or *P. heterophylla* Bunge, which has the functions of clearing away heat and toxic materials, eliminating dampness and checking vaginal discharge, stopping bleeding by astringency (5). Studies have shown that acetic ether extract of *P. herba* can inhibit the proliferation of human oral epithelial cancer, colon cancer, and gastric cancer and induce apoptosis, showing a certain antitumor activity (6). However, the effect of *P. herba* aqueous extract on the biological behavior of hepatocellular carcinoma cells and its mechanism has not yet been elucidated. Long non-coding RNA (lncRNA) is a group of RNA consisting of more than 200 nucleotides, which plays an important regulatory role in gene transcription, splicing and epigenetics, as well as biological processes involving cell cycle, development and pluripotency (7). Certain lncRNAs are found to be imbalanced in tumors, and their abnormal expression often helps drive or hinder tumor development (8-9). As an antisense RNA of human serotonin 2A receptor gene (HTR2A), HTR2A-AS1consisting of 18 exons is located on chromosome 13 (10). Data show that HTR2A-AS1 has down-regulated expression in liver cancer, showing a negative correlation with patient survival (11). However, the biological function and significance of HTR2A-AS1 in liver cancer remain to be revealed. Based on this, this study investigates the effect of *P. herba* aqueous extract on the proliferation, apoptosis, invasion and migration of hepatocellular carcinoma cells as well as its regulation of HTR2A-AS1, with a view to revealing the function of *P. herba* aqueous extract in liver cancer and its potential mechanism.

Materials and Methods

Main reagents

Hepatocellular carcinoma cell MHCC97-H was purchased from the Typical Culture Collection Center of the Chinese Academy of Sciences. Roswell Park Memorial Institute (RPMI) -1640 medium, and Lipofectamine ™ 2000 transfection reagents were purchased from Invitrogen, USA. Cell Counting Kit-8(CCK-8) was purchased from Dojindo, Japan. pcDNA3.1, pcDNA3.1-HTR2A-AS1, si-NC, si-HTR2A-AS1 were purchased from Guangzhou Ruibo Biological Technology Co., Ltd. P21 (Article No.: ab227443), Cysteinyl aspartate specific proteinase 3 (Caspase-3) (Article No.: ab2302), E-cadherin (Article No.: ab15148), Matrix metalloproteinase-2 antibody (MMP-2) (Article No.: ab37150), HRP-labeled secondary antibody (Article No.:ab6721) were purchased from Abcam, USA.

Preparation of *P. herba* aqueous extract was performed according to the method of Hu Xinxin (12). Take 50g *P. herba* powder, add distilled water at a ratio of 1:30, soak it for 30 min, and cook it for 1.5 h to obtain the supernatant. Further, add 500 mL distilled water, cook it for 1 h, repeat 2 times. The supernatants collected in the above 3 steps were combined, concentrated to 50 mL, and stored at -20 ° C until use.

Cell culture and processing

MHCC97-H cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin in 37°C, 5% CO_2 humidified cell incubator for routine culture. The MHCC97-H cells in logarithmic growth phase were treated with *P. herba* aqueous extract at concentrations of 2.5, 5, and 10 mg/mL, and recorded as drug group 1, drug group 2, and drug group 3, respectively. The normally cultured cells were set as a control group. After 48 h treatment, cells were collected for various tests.

Cell transfection and processing

MHCC97-H cells were seeded into a six-well plate at a density of 1×10^5 cells/well. When 80% confluence was reached, cell transfection was carried out using pcDNA3.1, pcDNA3.1-HTR2A-AS1, si-NC, si-HTR2A-AS1 plasmid via Lipofectamine ™ 2000 transfection reagent. Where, cells transfected with si-NC and si-HTR2A-AS1 were treated with *P. herba* aqueous extract at a concentration of 10 mg/mL. After 48 h, cells were collected for various tests.

CCK-8 detection of cell survival

MHCC97-H cells were seeded into a 96-well plate at a density of 5×10^4 cells/well for different treatments. After 48 h, 10 μL CCK-8 solution was added according to the manufacturer's protocol to determine cell survival. After 1 h, optical density (OD) was measured by a microplate reader at a wavelength of 450 nm to calculate cell survival rate (OD in the experimental group/OD in the control group x 100%).

Detection of apoptosis by flow cytometry

Apoptosis was evaluated using the Annexin V-FITC/propidium iodide (PI) apoptosis detection kit. 1×10^6 MHCC97-H cells were collected, sequentially added with 5 μL Annexin V-FITC and 5 μL PI to react at room temperature for 15 min under a shade, and the apoptosis was analyzed by flow cytometry.

Plate cloning experiment to detect cell clone formation

MHCC97-H cells were seeded in 60 mm petri dishes and cultured at 37°C for 10-14 d. When ≥50 cell colonies were formed, the cells were washed 3 times with phosphate buffer, fixed with methanol for 15 min and stained using Giemsa for 20 min, and the number of cell clones was counted.

Western blot detection of P21, Caspase-3, E-cadherin, MMP-2 protein expression

The cell lysate was prepared using RIPA buffer, the total protein of MHCC97-H cells was extracted, and the protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein was transferred to a polyvinylidene fluoride (PVDF) membrane for 1 h at a constant current of 400 mA. The PVDF membrane was blocked with 5% skim milk for 30 min, and then incubated overnight with primary antibodies of P21, Caspase-3, E-cadherin, MMP-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (all diluted by 1: 1000) at 4°C. The membrane was then washed 3 times with Tris-HCl-Tween buffer salt solution (TBST), incubated with the secondary antibody for 30 min at room temperature and washed 3 times with TBST buffer. Protein bands were detected in a dark room using chemiluminescence.

qPCR detection of HTR2A-AS1 expression

MHCC97-H cells were collected, washed twice with Phosphate Buffered Saline (PBS), then total RNA was extracted using Trizol reagent. cDNA was synthesized using a reverse transcription kit, which was used as a template for the qPCR reaction. The experimental data were analyzed by $2^{-\Delta\Delta Ct}$. The primer sequence was HTR2A-AS1 5'-ACAGGACCTGACAACTCCAC-3'(F), 5'-CCATGCTACCGATGACTGGG-3'®. The internal reference GAPDH was 5'-GGAGCGAGATCCCTCCAAAAT-3'(F), 5'-GGCTGTTGTCATACTTCTCATGG-3'(R).

Detection of cell invasion and migration by Transwell chamber

For the invasion assay, the Transwell upper chamber was coated with Matrigel gel and allowed to stand at 37°C for 3-4 h. After different cell treatments, 200 μL MHCC97-H cells (2.5×10^4/mL serum-free RPMI-1640 medium) were seeded into the upper chamber, and 750 μL RPMI-1640 medium containing 10% FBS was added to the lower chamber as a chemical attractant. After 24 h incubation, uninvaded cells were scraped off with a cotton swab. The cells invaded the lower filter surface were washed twice in PBS, fixed with 4% formaldehyde for 15 min, and then stained with 0.25% crystal violet for 20 min. Under an optical microscope, cells invaded through the wells were counted.

For the migration assay, Matrigel gel was not added to the Transwell upper chamber. The other experimental steps were the same as those in the invasion experiment.

Statistical Analysis

SPSS 22.0 software was used for statistical analysis of the data, and the results were expressed as mean ± standard deviation(x±s). t-test was used to compare the differences between the two groups, one-way analysis of variance was used to compare the differences between multiple groups, and SNK-q test was used to perform multiple comparisons between groups. The difference was considered statistically significant when $P<0.05$.

Results

Effect of *P. herba* aqueous extract on MHCC97-H proliferation and apoptosis

CCK-8, flow cytometry, plate cloning and western blot testing results are shown in Table 1 and Figure 1. Compared with the control group, 2.5, 5, 10 mg/mL *P. herba* aqueous extract significantly reduces MHCC97-H cell survival rate, significantly increases the cell apoptosis rate, decreases the number of formed clones, and increases P21 and Caspase-3 protein expressions ($P<0.05$), all in a concentration-dependent manner.

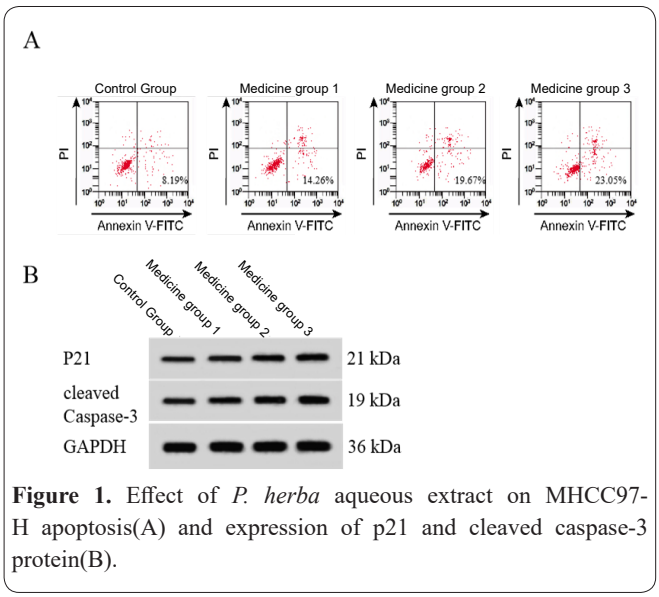

Figure 1. Effect of *P. herba* aqueous extract on MHCC97-H apoptosis(A) and expression of p21 and cleaved caspase-3 protein(B).

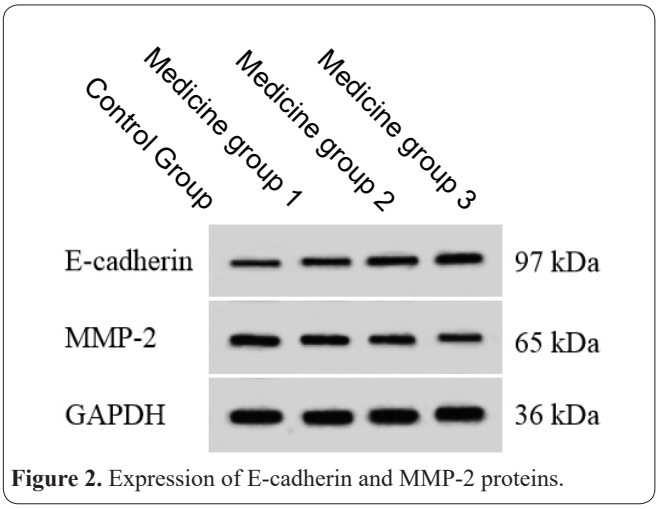

Figure 2. Expression of E-cadherin and MMP-2 proteins.

Effect of *P. herba* aqueous extract on MHCC97-H migration, invasion and HTR2A-AS1 expression

qPCR, western blot and Transwell testing results are shown in Table 2 and Figure 2. Compared with the control group, 2.5, 5, 10 mg/mL *P. herba* aqueous extract significantly increase HTR2A-AS1 expression and E-cadherin protein expression in MHCC97-H cells, significantly reduce MMP-2 protein level and reduce the number of migrating cells and invading cells ($P<0.05$), all in a concentration-dependent manner.

Effect of HTR2A-AS1 overexpression on the proliferation and apoptosis of MHCC97

CCK-8, flow cytometry, plate cloning and western blot testing results are shown in Table 3 and Figure 3. Compared with the pcDNA3.1 group, HTR2A-AS1overexpression significantly reduces the survival rate of MHCC97-H cells, significantly improves the cell apop-

Table 1. Effects of *P. herba* aqueous extract on MHCC97-H proliferation and apoptosis($\bar{x}$±s, n=9).

Group	Survival rate (%)	Apoptosis rate (%)	Number of formed clones	P21	Caspase-3
Control group	100.02±8.17	8.19±1.01	138±8.14	0.21±0.02	0.28±0.03
Drug group 1	85.67±7.24*	14.26±1.37*	110±7.25*	0.32±0.02*	0.41±0.03*
Drug group 2	71.06±6.59*	19.67±1.59*	81±6.96*	0.46±0.03*	0.64±0.04*
Drug group 3	54.53±5.41*	23.05±1.82*	66±6.15*	0.67±0.04*	0.81±0.05*
F	71.463	174.219	178.740	429.456	340.271
P	0.000	0.000	0.000	0.000	0.000

Drug group 1: *P. herba* aqueous extract 2.5 mg/mL; Drug group 2: *P. herba* aqueous extract 5 mg/mL; Drug group 3: *P. herba* aqueous extract 10 mg/mL. Note: Compared with the control group,*$P<0.05$.

Table 2. Effect of *P. herba* aqueous extract on MHCC97-H migration and invasion($\bar{x}$±s, n=9).

Group	HTR2A-AS1	E-cadherin	MMP-2	Number of migrating cells	Number of invading cells
Control group	1.01±0.06	0.22±0.02	0.76±0.06	138±9.45	84±8.25
Drug group 1	1.31±0.07*	0.34±0.03*	0.64±0.05*	120±9.14*	70±7.15*
Drug group 2	1.64±0.08*	0.57±0.04*	0.40±0.04*	96±8.82*	56±6.27*
Drug group 3	1.89±0.09*	0.69±0.05*	0.31±0.03*	75±7.11*	43±5.35*
F	230.752	304.222	181.779	90.632	60.202
P	0.000	0.000	0.000	0.000	0.000

Note: Compared with the control group, *$P<0.05$.

tosis rate, reduces the number of formed clones, and increases P21 and Caspase-3 protein expressions (P <0.05).

Effect of HTR2A-AS1 overexpression on migration and invasion of MHCC97

qPCR, western blot and Transwell testing results are shown in Table 4 and Figure 4. Compared with the pcDNA3.1 group, HTR2A-AS1 overexpression significantly increases HTR2A-AS1 expression and E-cadherin protein expression in MHCC97-H cells, significantly reduces MMP-2 protein levels, and reduces the number of migrating cells and invading cells (P <0.05).

Effect of HTR2A-AS1 inhibition on proliferation and apoptosis of *P. herba* aqueous extract-treated MHCC97-H

CCK-8, flow cytometry, plate cloning and western blot testing results are shown in Table 5 and Figure 5. Compared with drug group 3 + si-NC group, HTR2A-AS1 inhibition significantly increases the survival rate of *P. herba* aqueous extract-treated MHCC97-H cells, significantly reduces cell apoptosis rate, increases the number of clones formed in the cells, and reduces P21 and Caspase-3 protein expressions(P<0.05).

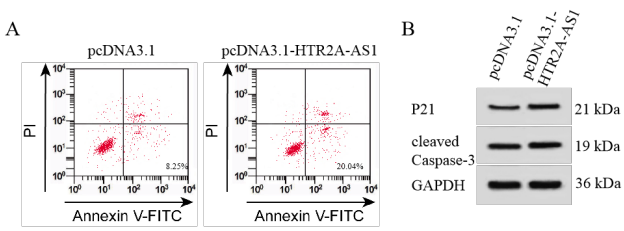

Figure 3. Effect of HTR2A-AS1 over-expression on MHCC97a-poptosis and P21 and Caspase-3 expressions A: Effect of HTR2A-AS1 over-expression on MHCC97 apoptosis; B: P21 and Caspase-3 protein expressions.

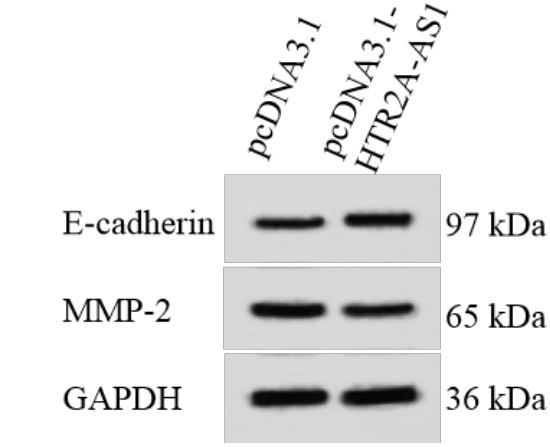

Figure 4. E-cadherin and MMP-2 protein expressions.

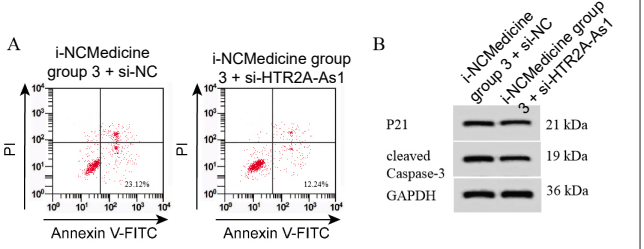

Figure 5. Effect of HTR2A-AS1 inhibition on MHCC97-H apoptosis and P21 and Caspase-3 expression in *P. herba* aqueous extract A: Effect of HTR2A-AS1 inhibition on apoptosis in *P. herba* aqueous extract-treated MHCC97-H; B: P21 and Caspase-3 protein expressions.

Table 3. Effect of HTR2A-AS1 over-expression on the proliferation and apoptosis of MHCC97 (x±s, n=9).

Group	Survival rate (%)	Apoptosis rate (%)	Number of formed clones	P21	Caspase-3
pcDNA3.1	100.00±8.18	8.25±1.10	136±8.07	0.22±0.02	0.30±0.03
pcDNA3.1-HTR2A-AS1	56.21±6.13*	20.04±1.59*	71±7.00*	0.55±0.03*	0.71±0.04*
t	12.852	18.294	18.253	27.458	24.600
P	0.000	0.000	0.000	0.000	0.000

Note: Compared with pcDNA3.1 group,*P<0.05.

Table 4. Effect of HTR2A-AS1 over-expression on migration and invasion of MHCC97 ($\bar{x}$±s, n=9).

Group	HTR2A-AS1	E-cadherin	MMP-2	Number of migrating cells	Number of invading cells
pcDNA3.1	1.02±0.05	0.23±0.02	0.73±0.05	139±9.28	85±7.94
pcDNA3.1-HTR2A-AS1	2.68±0.07*	0.61±0.03*	0.34±0.03*	82±8.31*	49±5.02*
t	57.891	31.618	20.065	13.727	11.497
P	0.000	0.000	0.000	0.000	0.000

Note: Compared with pcDNA3.1 group,*P<0.05.

Table 5. Effect of HTR2A-AS1 inhibition on proliferation and apoptosis of *P. herba* aqueous extract-treated MHCC97-H ($\bar{x}$±s, n=9).

Group	Survival rate (%)	Apoptosis rate (%)	Number of formed clones	P21	Caspase-3
Drug 3 group +si-NC	54.62±5.48	23.12±1.88	67±6.60	0.68±0.04	0.80±0.05
Drug 3 group +si-HTR2A-AS1	89.28±6.92*	12.24±1.25*	120±8.92*	0.30±0.03*	0.36±0.04*
t	11.780	14.478	14.329	22.800	20.615
P	0.000	0.000	0.000	0.000	0.000

Note: Compared with drug 3 group + si-NC group, *P<0.05.

Table 6. Effect of HTR2A-AS1 inhibition on migration and invasion of *P. herba* aqueous extract-treated MHCC97-H ($\bar{x}$±s, n=9).

Group	HTR2A-AS1	E-cadherin	MMP-2	Number of migrating cells	Number of invading cells
Drug 3 group +si-NC	1.03±0.05	0.68±0.05	0.30±0.03	74±7.15	44±5.33
Drug 3 group +si-HTR2A-AS1	0.23±0.03*	0.33±0.03*	0.66±0.04*	117±8.18*	70±6.91*
t	41.160	18.007	21.600	11.874	8.938
P	0.000	0.000	0.000	0.000	0.000

Note: Compared with drug group 3+ si-NC group,*P<0.05.

Effect of HTR2A-AS1 inhibition on migration and invasion of *P. herba* aqueous extract-treated MHCC97-H

The results of qPCR, western blot and the Transwell test are shown in Table 6 and Figure 6. Compared with drug group 3 + si-NC group, HTR2A-AS1 inhibition significantly reduces HTR2A- AS1 expression and E-cadherin protein expression in MHCC97-H, significantly increases MMP-2 protein level, and increases the number of migrating cells and invading cells (P<0.05).

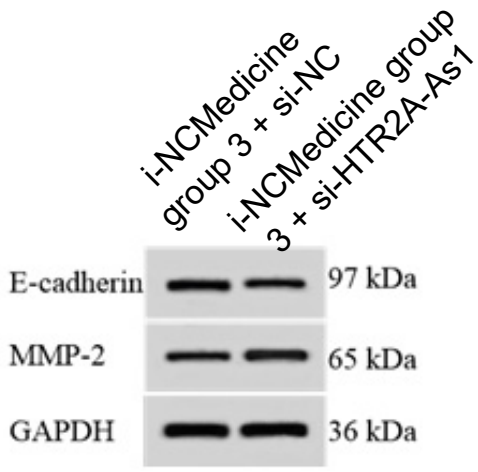

Figure 6. E-cadherin and MMP-2 protein expressions.

Discussion

As one of the most lethal and highly aggressive malignancies, liver cancer seriously endangers people's health (13). Cancer indicates an abnormal progression of tissue cells and uncontrolled proliferation (14). Therefore, understanding the malignant biological behavior of hepatocellular carcinoma cells can help us develop novel therapeutic strategies. In this study, it was detected that *P. herba* aqueous extract could inhibit the proliferation, migration and invasion of hepatocellular carcinoma cells and promote its apoptosis. It is worth noting that *P. herba* aqueous extract adds HTR2A-AS1 expression in hepatocellular carcinoma cells. Therefore, the antitumor function of *P. herba* aqueous extract may be closely related to the regulation of HTR2A-AS1 expression. Moreover, HTR2A-AS1 may be an important diagnostic biomarker and a potential target for liver cancer treatment.

As a traditional Chinese medicine, *P. herba* has rich chemical constituents such as saponins, lignans, iridoids, polysaccharides, which demonstrates extensive pharmacological activities in antitumor, immune regulation, antibiosis and antiviral (15). Data show that *P. herba* aqueous extract promotes the cycle arrest of leukemia HL-60 cells and K562 cells (16), and inhibits the proliferation and induces apoptosis of K562 cells (12). *P. herba* aqueous extract at different concentrations can inhibit tumor growth of cervical cancer-bearing mice (17), and alcohol extracts of *P.* scabra Bunge show strong cytotoxic activity against human cervical cancer HeLa cells and gastric cancer MNK-45 cells (18). In this study, *P. herba* aqueous extract significantly reduced the survival rate, colony formation, number of migrating cells, number of invading cells, and MMP-2 protein expression in hepatocellular carcinoma cell MHCC97-H in a concentration-dependent manner, and significantly increased cell apoptosis, P21, Caspase-3, and E-cadherin protein expression levels, suggesting that treatment using *P. herba* aqueous extract inhibits the proliferation, migration, and invasion of hepatocellular carcinoma cells, and promotes its apoptosis. *P. herba* aqueous extract thus exhibits certain anti-hepatoma activity.

The biological function and significance of lncRNA in the occurrence and development of cancer have been widely reported (19). In liver cancer, different lncRNAs act as oncogenes or tumor suppressors which regulate tumor progression (20). For example, MCM3AP-AS1 is a new type of oncogenic lncRNA, inhibition of its expression can inhibit proliferation, colony formation and cell cycle progression of hepatocellular carcinoma cells, and induce apoptosis in vitro (21). DGCR5 has down-regulated expression in hepatocellular carcinoma cells, whose overexpression can significantly inhibit the growth, migration and invasion of hepatocellular carcinoma cells (22). As one of the lncRNAs, HTR2A-AS1 plays a key role in the occurrence and development of liver cancer. Liao et al. (23) found via data analysis based on The Cancer Genome Atlas (TCGA) that compared with adjacent normal liver tissue, primary tumors in patients with liver cancer had lowly-expressed HTR2A-AS1, which can be used as a potential biomarker for liver cancer diagnosis and prognosis. This study found that *P. herba* aqueous extract significantly reduced HTR2A-AS1 expression in hepatocellular carcinoma cell MHCC97-H in a concentration-dependent manner, suggesting that *P. herba* aqueous extract's role in resisting liver cancer may be related to the regulation of HTR2A-AS1 expression. The subsequent functional experiment results indicated that HTR2A-AS1 overexpression significantly reduced the survival rate, colony formation, number of migrating cells, number of invading cells, and MMP-2 protein level of hepatocellular carcinoma cells, while significantly increasing cell apoptosis rate, P21, Caspase-3, E-cadherin protein levels and HTR2A-AS1 expression levels. It suggests that HTR2A-AS1 acts as a tumor suppressor gene in liver cancer, which can inhibit the survival, clone for-

mation, migration and invasion of hepatocellular carcinoma cells, and induce cell apoptosis. Further investigation into the action mechanism of *P. herba* aqueous extract showed that HTR2A-AS1 inhibition significantly increased the survival rate, colony formation, number of migrating cells, number of invading cells and MMP- 2 protein expression of *P. herba* aqueous extract-treated MHCC97-H cells, but significantly reduced the cell apoptosis rate, P21, Caspase-3, E-cadherin protein levels, indicating that *P. herba* aqueous extract's inhibitory effect on the proliferation, migration, and invasion of hepatocellular carcinoma cells and its promotion effect on apoptosis of hepatocellular carcinoma cells were reversed by HTR2A-AS1 inhibition. Combining the foregoing results, it was confirmed that the antitumor effect of *P. herba* aqueous extract on liver cancer may be achieved by the regulation of HTR2A-AS1 expression.

To sum up, *P. herba* aqueous extract can effectively inhibit the proliferation, migration and invasion of hepatocellular carcinoma cells, and induce its apoptosis. Its mechanism of action is closely related to the regulation of HTR2A-AS1 expression in hepatocellular carcinoma cells, which provides new enlightenment for the development and utilization of drugs for liver cancer treatment.

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