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Original Article

Impact analysis of huaier extract on cell proliferation of bladder tumor cells and Wnt/β-catenin signal pathway

Tiejun Liang, Faren Xu, Xiaopeng Zhang, Nanhui Zhang, Nannan Yang

Department of Urinary Surgery, Xinchang People's Hospital, Shaoxing, Zhejiang, China

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Abstract: Objective: To investigate the inhibitory effect and mechanism of huaier extract on cell proliferation of human bladder cancer. Methods: 42 mice were selected to establish mice models of bladder cancer cells (T24) and were randomly divided into two groups as the experimental group (infusing Huaier extract by gastrogavage) and the control group (infusing saline by gastrogavage). MTT method was used to detect the difference of cancer cells' proliferation in these two groups, and compare the difference of xenograft tumor volume between two groups. Enzyme-linked Immunosorbent Assay (ELISA) was used to detect differences in expression levels of VEGF, bFGF, IL-1 and TNF-α; flow cytometry was used to detect the difference of drug effect on cell apoptosis and cycle; and RT-PCR method was used to detect the expression difference of Wnt, β-catenin, E-cadherin, and N-cadherin in two groups. Results: Huaier extract had a significant inhibitory effect on cancer cell proliferation in a dose-dependent manner. 20 days after modeling, xenograft tumor volume of the experimental group was significantly lower than that of the control group ($P<0.05$). Compared to control group, the expression levels of VEGF and bFGF in the experimental group were obviously lower, while IL-1 and TNF-α were significantly higher ($P<0.05$). And the early apoptosis rate of cells and proportion of cells in S stage in experimental group were also significantly higher than those in control group ($P<0.05$). Compared to control group, the ΔCT values of Wnt, β-catenin and N-cadherin in the experimental group were significantly higher, while E-cadherin was significantly lower ($P<0.05$). Conclusion: Huaier extract could significantly inhibit the cell proliferation of human bladder cancer and it was related to the effect of Wnt/β-catenin signal pathway.

Keywords: Huaier extract, bladdercancer, cell proliferation, signal pathway

Introduction

Clinical statistics showed that urothelial carcinoma was the most common malignant tumor in the urinary system, and the incidence had been rising [1]. Due to rich blood vessels and lymphatic vessels in the lamina propria of the bladder, invasive migration and distant metastasis easily occurred in the bladder cancer, which led to the loss of ideal timing for surgery when the most patients were diagnosed with bladder cancer, and the combined chemotherapy was mostly performed in these patients [2]. However, due to high incidence of side effects of chemotherapy, poor tolerance of patients and high mortality, it had important clinical implications to explore a new therapy for bladder cancer. Huaier extract is a hot water extract from fermented Huaier (a kind of medical fungi), and its main anticancer active constituent is polysaccharide protein [3]. Huaier extract has been widely used in the treatment of several malignant tumors clinically. Studies showed that Huaier extract could rise organism's anticancer immune surveillance function, promote organism to produce cytokine, inhibit the emergence of tumor angiogenesis, induce apoptosis in malignant tumor cells and reduce multidrug resistance genes expression level [4-6]. However, without definite antineoplastic mechanism and experimental research reports about inhibition of Huaier extract on bladder cancer cells, the relating research achievements have been quite limited so far. Therefore, in this study, the human bladder tumor T24 cell line was inoculated into nude mice to establish bladder tumor metastasis model in nude mice. The nude mice were treated with peritoneal injection of Huaier extract. The observation of the proliferation of tumor cells and exploration

of its related mechanism were conducted in order to provide a further experimental basis for the treatment of bladder cancer with Huaier extract.

Materials and methods

Experimental materials

The human bladder cancer cell line (T24) was purchased from the Cell Bank of the Chinese Academy of Sciences. 42 male BALB/c athymic nude mice weighted (21±3) g and aged 5 to 6 weeks were purchased from Beijing Weitong Lihua Experimental Animal Technology Co.

10% fetal bovine serum, RPMI1640 culture solution and trypsin were purchased from Gibco, USA; tetrazolium blue (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma, USA; the Trametes Huaier extract was purchased from Qidong Gaitianli Pharmaceutical Co, solution of 200 mg/ml with saline as solute was sterilized with 20 μm filter membrane, and kept for using in 4°C; vascular endothelial growth factor (VEGF), Basic fibroblast growth factor (BFGF), Interleukin-1 (IL-1) and Enzyme-Linked Immunosorbent Assay (ELISA) kit of tumor necrosis factor-α (TNF-α) were purchased from R&D of USA. Annexin V-FITC/PI apoptosis detection kit and the cycle kit were purchased from the BD Company of USA. RNA extraction kit was purchased from Germany QIAGEN Company; cDNA reverse transcriptase kit and SYBR dye real-time fluorescence quantitative kit were purchased from Japanese TaKaRa Corporation. Wellscan Mk3 microplate reader was purchased from Thermo Fisher Scientific, USA. RT-PCR instrument was purchased from Applied Biosystems.

Establishment of bladder cancer animal model of lymph node metastasis

The T24 cell line of human bladder tumor was incubated with RPMI1640 culture solution containing 10% fetal bovine serum at 37°C in atmosphere of 5% CO_2. When the tumor cells were in the logarithmic growth phase, the cells were washed and digested to form single cell suspension solution with a concentration of 5 * 10^7/ml, and then performed the subcutaneous injection in the abdomen of nude mice under aseptic condition within 30 min, the dose of 50 μl/only. After two weeks of modeling, the growth of human bladder tumor T24 cells in the subcutaneous abdomen of the nude mice was observed every 5 days, and the volume was calculated and recorded. The relative volume was calculated as: π/6 (length * width * thickness).

Grouping

After modeling, the 42 mice were randomly divided into two groups according to the random number table. The mice in the experimental group were infused with the solution of Huaier extract in the stomach mice at a dose of 5 g/(kg * 2 d). And in the control group, the stomach of mice was infused with normal saline at a dose of 0.5 ml/2 d. Each group has 21 modeled nude mice. And there was no significant difference in age, body weight, diet and other factors between the two groups (P>0.05).

MTT assay

The T24 cells in logarithmic growth phase were prepared into cell suspension at a concentration of 5 * 10^3/ml and inoculated in 96-well culture plates at a volume of 100 μl each plate. After incubation for 12 hours, the medium was removed and the culture medium containing Huaier extract at concentration of 200 mg/ml was added to each well in experimental group. The final concentration of the drug was regulated into 0.5, 1, 2, 5, 10, 20, 40 and 80 mg/ml, respectively. The normal saline was added to the control group. Three sets of parallel hole were set up in each group. After incubation for 36 h, the cells were incubated with 20 μl 0.5% MTT solution and the absorbance (OD values) was measured at 570 nm using a microplate reader. The inhibitory rate was calculated according to the following equation: Cell Proliferation Inhibition = (OD in control group-OD in experimental group)/OD in control group * 100%.

Detection of serum-associated factors

Venous blood was collected from the vein of nude mice tail in two groups. The serum expression levels of VEGF, bFGF, IL-1 and TNF-α were detected by ELISA in nude mice of two groups, and the differences of expression level were compared between two groups.

Detection of cell apoptosis and cell cycle

Logarithmic phase T24 cell were collected and inoculated to six-well plates, 2 ml and 1 * 10^6

Table 1. The sequence of synthetic primer

Gene symbol		Sequence
β-catenin	Up stream	5'-TGAGCGGGCTACAGCTT-3'
	Down stream	5'-TCCTTAATGTCACGCACGATTT-3'
Wnt	Up stream	5'-'GCACAACAGAGAATGCCAGC-3'
	Down stream	5'-GACGGTTGGATCTGCCATGA-3'
E-cadherin	Up stream	5'-TGCCCAGAAAATGAAAAAGG-3'
	Down stream	5'-GTGTATGTGGCAATG CGTTC-3'
N-cadherin	Up stream	5'-ACAGTGGCCACCTACAAAGG-3'
	Down stream	5'-CCGAGATGGGGTTGA TAATG-3'

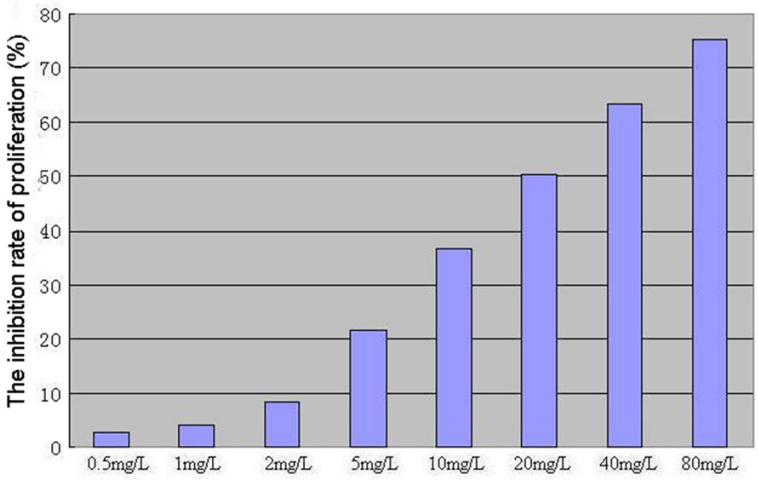

Figure 1. The inhibitions rate that different concentration of Huaier extract to cancer cell.

T24 cells per well. Two groups were divided and incubated with extract of Huaier and normal saline respectively for 24 hours, and then the cells were collected. After putting and mixing 5 μl Annexin V-FITC, 15 minutes of incubation in the dark in room temperature was conducted, 5 μl PI was put into the two groups before the machine, apoptotic rate of cell of two groups was detected by flow cytometry.

1 * 10^6 logarithmic phase cells (T24) were collected and inoculated to culture flasks, two groups were divided and incubated with extract of huaier and saline for 24 hours, then the cells were collected. According to the description of cell cycle kit, flow cytometry was applied to detect the proportion of cells which were in the cell cycle.

Detection of signal pathway molecules

The cancer tissues were taken from two groups' nude mice and the total RNA was extracted by using RNA kits. The OD value was measured at 260 nm and 280 nm respectively by ultraviolet spectrophotometer and then the content and purity of RNA were calculated. Afterwards, with the application of cDNA reverse transcriptase kit, 3 μg RNA was reversed into cDNA, and then cDNA was regarded as template to perform PCR amplification. All PCR primers, whose internal control was β-Actin, were synthesized by Shanghai Biological Engineering Co. Ltd. The expression amount of mRNA of Wnt, β-catenin, E-cadherin and N-cadherin in two groups were detected by RT-PCR methods, and the differences were compared between the two groups. The primer designed and synthesized by Shanghai Biological Engineering Co. Ltd, the primer sequence is as follows, see **Table 1**. PCR reaction condition: 95°C predegenerated 30 s, 95°C denature 5 s, 55°C annealing 15 s, 72°C extension 45 s, a total of 40 cycles.

Statistics processing

The statistical analyses were performed by SPSS 17.0. Measurement data were expressed by $\bar{x} \pm S$ and group t-test was used for the comparison between groups. The count data was expressed by percentage, and χ^2 was taken for the comparison between groups. $P<0.05$ was considered with statistical significance.

Results

Comparison of the inhibition rate of cancer cells in the two groups

Huaier extract with different concentration had inhibitory effect on tumor cell proliferation and the inhibitory effect was proportional to the concentration of Huaier extract and in a dosage-dependence manner (χ^2=10.246, P= 0.009). The inhibitory effect of Huaier extract was not significant when the concentration was below 5 mg/ml (P>0.05), see **Figure 1**.

Table 2. Comparison of the levels of serum related-factors in two groups of nude mice (pg/ml)

Group	VEGF	bFGF	IL-1	TNF-α
Study group	25.6±3.2	17.6±4.2	85.9±12.4	68.2±9.5
Control group	47.9±8.4	39.2±6.8	37.1±8.7	22.6±5.9
T value	3.241	3.355	3.854	4.013
P value	0.025	0.010	0.004	0.001

Table 3. Comparison of cell apoptosis and cell cycle in two groups of nude mice (%)

Group	Cell apoptosis	G0/G1 stage	G2/M stage	S stage
Study group	4.61±0.76	21.8±6.6	32.6±8.2	51.4±12.1
Control group	1.59±0.24	30.3±7.4	33.1±8.5	32.9±9.6
t/χ^2 value	4.371	2.831	1.518	3.957
P value	0.001	0.039	0.082	0.002

Comparison of the levels of serum related-factors of nude mice in two groups

Compared to the control group, the expression level of serum VEGF and bFGF in the experimental group was significantly lower, but the expression level of IL-1 and TNF-α was significantly higher, (P<0.05) see **Table 2**.

Comparison of cell apoptosis and cell cycle of nude mice in two groups

After 48 hours of treatment of tomor cells with 11 mg/mL Huaier extract in the experimental group, early apoptosis rate and the ratio of cells in S stage were significantly higher than control group (P<0.05). And the ratio of cells in G2/M stage of two groups had no apparent difference (P=0.082), see **Table 3** and **Figure 2**.

Comparison of the expression level on signal pathway molecules of nude mice in two groups

In the experimental group, the ΔCT value of Wnt, β-catenin and N-cadherin in tumor tissues was significantly higher than that in the control group while the ΔCT value of E-cadherin was remarkably lower than that in the control group (P<0.05), as shown in **Table 4**.

Observation and measurement of the growth of subcutaneous transplanted tumor of nude mice

The volume growth rate of subcutaneous transplanted tumor in the control group was significantly higher than that in the experimental group. There was no obvious difference of the volume of subcutaneous transplanted tumor between two groups on the 15th day after the modeling (P>0.05). The volume of the tumor in the experimental group was significantly smaller than that in the control group since the 20th day after the modeling (P<0.05), as shown in **Table 5**.

Discussion

At present, patients with bladder cancer are often treated with chemotherapy after surgery [5]. Although there are major chemotherapy drugs for bladder cancer, many complications still exist in these drugs and compliance of patients is poor. Therefore, it is necessary to choose appropriate drugs for adjunctive therapy to inhibit the growth and recurrence of tumor. Studies on clinical pharmacology prove that Huaier extract can inhibit the growth, proliferation and metastasis of malignant tumors [6, 7]. In recent years, it has been confirmed by some animal experiments that Huaier extract can significantly prolong the survival time of cancer bearing Mice [8, 9]. However, there are little research about whether Huaier extract has effective inhibitory effects on the proliferation and mechanism of action of bladder cancer. Therefore, this study used bladder cancer cell line T24 as the object of study to explore the biological effects and related mechanism of Huaier extract in the bladder carcinoma cells.

This study had constructed the nude mouse models of Bladder Cancer T24 Cells and analyzed its inhibitory effects on the proliferation of tumor cells by infusing Huaier extract into the stomach of tumor bearing nude mice. The results showed that Huaier extract can remarkably inhibit the growth of tumor cells of the tumor bearing nude mice and had clear anticancer effects, which were consistent with the already proved inhibitory effects of Huaier extract on the proliferation and metastasis of liver cancer and other malignant tumors [10-13]. This study also used Huaier extract with different drug concentration to conduct the pro-

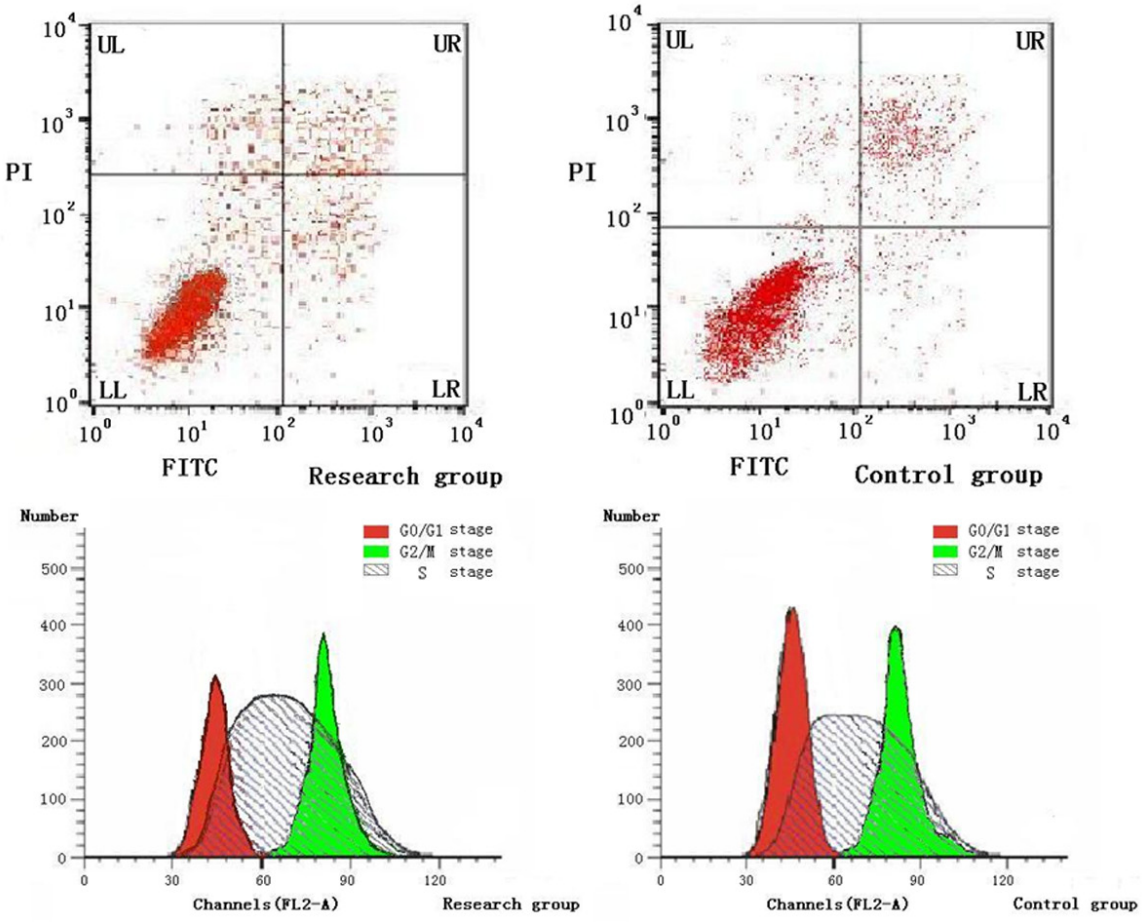

Figure 2. Analysis diagram of flow cytometry: influence of Huaier extract on cell apoptosis and cell cycle of nude mice.

Table 4. Comparison of the expression on signal pathway molecules (ΔCT value) between two groups of nude mice

Groups	Wnt	β-catenin	N-cadherin	E-cadherin
Study group	13.39±1.44	11.76±1.87	13.32±2.12	7.27±0.85
Control group	8.97±1.17	9.04±1.08	7.86±0.96	12.56±1.39
T value	3.214	2.726	3.423	3.326
P value	0.031	0.041	0.020	0.026

liferation tests of T24 cells respectively. The results showed that the inhibitory effects of Huaier extract on bladder carcinoma cells were dose-dependent and closely related to the Huaier extract concentration. Besides, when the Huaier extract concentration was over 5 mg/ml, it had remarkable inhibitory effects on bladder carcinoma cells.

VEGF and bFGF are both cytokines secreted by tumor cells. Their main functions are to promote the tumor angiogenesis and invasion metastasis by facilitating the proliferation and migration of endothelial cells and forming the new vessels. Meanwhile, serum IL-1 and serum TNF-α are mainly produced and released by macrophage. Serum IL-1 can kill tumor cells and induce their apoptosis while TNF-α can cause T lymphocytes to produce Correlation factors for killing tumor cells directly.

According to this study, the expression level of VEGF and bFGF of athymic mice infused with Huaier extract in stomach was notably lower, while the expression level of IL-1 and TNF-α was notably higher, indicating that the Huaier extract could restrain the generation and metastasis of tumor neoangiogenesis effectively by preventing tumor cells secreting VEGF and bFGF, refraining the generation of tumor

Table 5. Comparison of the growth and volume of subcutaneously xenotransplanted tumor between two groups of nude mice (mm^3)

Group	After the modeling (15 days)	After the modeling (20 days)	After the modeling (25 days)	After the modeling (30 days)
Study group	119.7±38.2	142.1±26.7	203.6±42.1	312.3±77.4
Control group	124.3±40.5	251.8±52.1	637.5±99.4	1737.5±142.5
T value	2.252	2.989	4.465	4.864
P value	0.064	0.033	0.001	0.001

neovessels, and strengthening macrophage to kill tumor cells.

Studies had confirmed that one of the most common mechanism of action of antitumor drugs was apoptosis and its impacts on cell cycles. Many laboratory animal studies had confirmed that the Huaier extract could induce the apoptosis of polytype malignant tumor, and then perform its cytotoxicity effect [11, 12]. According to this study, T24 cells, which were incubated with Huaier extract, the early apoptotic rate and the ratio of cells in S stage of were remarkably higher. That was to say, the Huaier extract, to a large extent, could induce the apoptosis of cells and block bladder tumor cells effectively in the S stage of cell cycle.

In recent years, plenty of studies had indicated that multiple signal pathways were involved in the process of the epithelial mesenchymal transition where epithelial carcinoma cells developed, and the Wnt and β-catenin signal pathways were the focus of the concern [16-18]. The up-regulation of tissue stromal related marker, such as Wnt, β-catenin and N-cadherin, and the down-regulation of epithelial related marker could cause the loss of cellular polar of epithelial carcinoma cells, the recombination of cytoskeletal protein within cells, gradual loosening of connections among cells and the declination of the adhesion of tumor cells, the dramatic increase of the ability of locomotory movement, invasion in neighboring tissues or transferring to distant site in vivo following hematogenous metastasis and lymph, which eventually led to the multiple metastasis of tumors [19, 20]. According to this study, after infusing Huaier extract into stomach of athymic mice in experimental group, related markers of Wnt, β-catenin and N-cadherin in their tissue of tumor decreased, while markers of E-cadherin increased, which proved the effective restraints effects of the Huaier extract on Wnt and β-catenin signal pathways. That was to say, the Huaier extract restrained Wnt signals notably, preventing the admission and accumulations of β-catenin in nuclei and then the activation of intranuclear target gene could be decreased, resulting in the decline of N-cadherin expression. And the rise in E-cadherin expression could enhance the cell adhesion and inhibit the metastasis of tumor cells. This result could also be verified from higher rate of volume increase of subcutaneous transplanted tumor in control group compared with experimental group. Meanwhile, the study revealed the inhibitory action of the Huaier extract on subcutaneous transplanted tumor of nude mice exerted its effects gradually twenty days after modeling.

To sum up, this study verified the effective inhibitory action of the Huaier extract on proliferation of bladder tumor cells. As the occurrence and development of bladder cancer is a complex process, involving multiple factors, the inhibitory mechanism of action of the Huaier extract is multifaceted, including preventing bladder tumor cells secreting VEGF and bFGF, up-regulating the expression level of IL-1 and TNF-α, inducing the apoptosis of bladder neoplasms and affecting Wnt and β-cateninsignal pathways and so on. However, only one type of T24 cell line was selected in this study, therefore, further clinical studies with large cell types are clearly needed for the further exploration of the inhibitory action and mechanism of Huaier extract on bladder neoplasms.

Disclosure of conflict of interest

None.

Address correspondence to: Tiejun Liang, Department of Urinary Surgery, Xinchang People's Hospital, No. 117 Gushan Middle Road, Shaoxing 312500, Zhejiang, P. R. China. Tel: +86-0575-86380823; Fax: 0575-86023271; E-mail: liangtiejunxinc8@163.com

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