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Original Article

Xiaoaiping improves the general state of rats with malignant ascites secondary to gastric cancer by blocking the TGF-β1 signaling pathway

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Abstract: Objective: To investigate the effect of Xiaoaiping on the general state of rats with malignant ascites secondary to gastric cancer by blocking the transforming growth factor-β1 (TGF-β1) signaling pathway. Methods: Fifty healthy Wistar rats were randomly divided into 5 groups, with 10 rats in each group. After successful modeling, 0.2 g/(20 g·d), 0.4 g/(20 g·d) and 0.8 g/(20 g·d) of Xiaoaiping injections were administrated at the low dose group (LDG), medium dose group (MDG) and high dose group (HDG), respectively. The model group (MG) was injected intraperitoneally with the same amount of sterile normal saline for 8 d. Rats in the control group (CG) were healthy without any treatment. The general states (mental state, dietary habits, reactions and body shape) of rats in each group were observed. The abdominal circumference, platelet (PLT) count, white blood cell (WBC) count, serum ferritin (SF), ascites volume, cell survival rate, and expression levels of TGF-β1 signaling pathway (TGF-β1, Smad2) were compared among the groups. Results: PLT and WBC counts in the MG were lower than those in the CG. Ascites volume, tumor cell survival rate, and SF in the MG were higher than those in the LDG, MDG and HDG ($P<0.05$). Thymus index in the LDG, MDG and HDG were significantly higher than that in the MG ($P<0.05$). There was no significant difference in the spleen indices among the groups ($P>0.05$). The kidney index, serum creatinine and urea nitrogen levels in LDG, MDG and HDG were significantly lower than those of MG ($P<0.05$). The LDG, MDG and HDG exhibited significantly higher peripheral blood CD4+ cells and CD4+/CD8+, and lower CD8+ level, as compared with the MG ($P<0.05$). The levels of serum interferon γ (IFN-γ), interleukin (IL)-1α, IL-1β, IL-2, IL-4 and tumor necrosis factor-α (TNF-α) in the LDG, MDG and HDG were higher than those in the MG ($P<0.05$). The ascites volume and tumor cell survival rate were decreased sequentially in the LDG, MDG and HDG ($P<0.05$). The MG had higher mRNA levels of TGF-β1 and Smad2 than the CG ($P<0.05$). No statistically significant difference was found in TGF-β1 and Smad2 between the LDG and MG ($P>0.05$). Conclusion: Xiaoaiping could significantly reduce the ascites volume in rats with gastric cancer, inhibit the production of tumor cells in the abdominal cavity, and improve the general state of rats in a dose-dependent manner. The mechanism may be related to the down-regulation of the mRNA level of TGF-β1 and Smad2.

Keywords: Transforming growth factor-β1, malignant ascites secondary to gastric cancer, Xiaoaiping, general state

Introduction

Cancerous ascites is a common complication of mid- to advanced-stage cancer, and malignancies that commonly cause ascites include ovarian cancer, malignant lymphoma of the abdomen, gastrointestinal malignancy, etc. Gastrointestinal malignancy has the worst prognosis, with survival of only 12-20 weeks, posing a great clinical challenge [1, 2]. In the past, radiotherapy and chemotherapy were used the most to treat malignant ascites secondary to gastric cancer, but the efficacy was not satisfactory, and the immune function of patients was weakened after treatment, seriously affecting their quality of life [3].

In recent years, using traditional Chinese medicine for the treatment of malignant tumors has attracted more attention. Chinese medicine has shown good curative effect in the treatment of malignant ascites secondary to gastric cancer. Combining Kanglaite injection with cisplatin intraperitoneal infusion, Zhang et al. [4]

successfully improved the control rate of malignant abdominal effusion of gastric cancer with spleen deficiency and dampness, reduced toxic and side effects, and improved TCM syndrome, which played a role of enhancing efficacy. Xiaoaiping, which is mainly made of Fissistigma glaucescens by modern technology, can suppress the growth of carcinoma cells and improve the immunity of patients. At present, it is widely used in the clinical treatment of lung cancer, liver cancer and esophageal cancer, with good inhibitory effect on cancer cells and high safety. However, its role and mechanism in malignant ascites secondary to gastric cancer have not been adequately clarified [5, 6]. Transforming growth factor (TGF)-β1 has been closely associated with tumor invasion, lymph node metastasis, immune cell activity, etc. while its effect on malignant ascites secondary to gastric cancer remains to be explored [7-9]. In this study, 50 healthy Wistar rats were randomly assigned to investigate the effects of Xiaoaiping on the general state of rats with malignant ascites secondary to gastric cancer.

Materials and methods

Experimental animals

Fifty healthy male Wistar rats (8-10 weeks of age, body weight 210-250 g) were supplied by SPF (Beijing) Biotechnology Co., Ltd. Animals were reared in separate cages at 20-26°C in a 12-h light/dark cycle, with water and food ad libitum. The animal license is SCXK (Beijing) 2011-0004.

Main instruments and reagents

The following instruments and reagents were used, RevertAidTM First-Strand cDNA Synthesis Kit (Shanghai Pengshuo Biotechnology Co., Ltd.); BGC823 gastric cancer cell line (Tongpai (Shanghai) Biotechnology Co., Ltd.); Trizol kit (Shanghai Runcheng Biotechnology Co., Ltd.); Xiaoaiping (Nanjing Shenghe Pharmaceutical Co., Ltd., Z20025868); PowerSYBRR Green PCRMaster Mix (ABI, USA); Centrifuge (EG162, Sheldon, USA).

Modeling and grouping

(1) Modeling methods [10]: BGC823 gastric cancer cell lines were subcultured to prepare viable cell suspension (1.0×10^7/mL), and 0.5 mL was subcutaneously inoculated into the inner thigh of the rat near the groin. After 7-8 d, the abdominal circumference of the rats increased significantly. Each rat was considered to be successfully modeled with malignant ascites if ascites was withdrawn by bilateral abdominal puncture. (2) Grouping methods: the 50 healthy rats were randomized into 5 groups. After successful modeling, 0.2 g/(20 g·d), 0.4 g/(20 g·d) and 0.8 g/(20 g·d) doses of Xiaoaiping injections were administered intraperitoneally for 8 d to the low dose group (LDG), medium dose group (MDG) and high dose group (HGD), respectively. The model group (MG) was administered intraperitoneally with equal amount of sterile normal saline for 8 d. The control group (CG) consisted of healthy rats without any treatment. Every procedure involving animal experiments was reported to and approved by the Animal Care and Use Committee of the First People's Hospital of Fuyang District.

Blood specimens

Twenty-four hours after drug administration on the last day, 1 mL of blood from abdominal aorta was taken and anticoagulated with ethylenediaminetetraacetic acid disodium (EDTA-Na2), and the counts of white blood cells (WBC) and platelets (PLT) were determined using MEK-6318K automatic blood cell analyzer (Nihon Kohden Corporation, Tokyo, Japan) and related reagents. In addition, venous blood (3 mL) was extracted from the caudal vein of rats and left for 10 min. After centrifugation, the serum was taken, and the serum ferritin (SF) level was measured according to the procedure of the kit (Hefei Zhihong Bio-Technology Co., Ltd., China). The testing equipment was DG5033A automatic microplate reader (Nanjing Huadong Electronics Group Medical Equipment Co., Ltd., China).

Ascites volume and cell survival rate

Twenty-four hours after drug administration on the last day, the rats were executed by cervical dislocation, and the abdomen was opened under aseptic conditions. The ascites was collected by pipetting using a centrifugal tube. The volume of ascites in each group was measured. Then, 1 mL of ascites sample was centrifuged and washed with phosphate buffer saline (PBS) for 2 times to prepare a cell solution (1×10^6/mL). Nine drops were pipetted and transferred

to an EP tube, followed by treatment with trypan blue solution (0.4%, 1 drop) and observation under a microscope. The dead cells were dyed light blue, and the cell viability (%) = the total number of viable cells/(the total number of dead cells + the total number of viable cells) × 100%. The cells in each flask were counted three times and averaged as the final value.

Thymus index, spleen index, kidney index and renal function

After the modeling, the rats were weighed and sacrificed by cervical dislocation. The thymus, spleen and kidney of rats were dissected and weighed to calculate the organ index, thymus index = weight of thymus/weight of rat, spleen index = weight of spleen/weight of rat, kidney index = weight of kidney/weight of rat. After collecting serum, the serum creatinine (Scr) and blood urea nitrogen (BUN) were measured using a whole blood automatic analyzer.

Peripheral blood T lymphocytes and related cytokines

After the modeling, 0.4 mL of blood was collected from tail vein and injected into an anticoagulant tube containing heparin, which was mixed for later use. The T-cell subsets CD4+, CD8+ and CD4+/CD8+ were measured using flow cytometry. The serum levels of interferon (IFN)-γ, interleukin (IL)-1α, IL-1β, IL-2, IL-4 and tumor necrosis factor (TNF)-α were determined using enzyme-linked immunosorbent assay (ELISA). ELISA kits were obtained from Shanghai Enzyme-Linked Biotechnology Co., Ltd., and the lot numbers were 180721, 171121, 181209 and 180103. The instructions of the kits were strictly followed.

Fluorescence quantitative PCR assay

Tumor cells were collected from rat ascites. RNA was extracted with Trizol reagent, and the absorbance at 260/280 nm was measured to analyze RNA concentration and purity. A reverse transcription kit was utilized to reverse transcribe RNA into cDNA, and PowerSYBRR Green PCRMaster Mix (2 ×) was used for PCR detection. The reaction conditions were as follows, 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 60 s. The upstream and downstream primers are as follows, TGF-β1: 5'-ATTCCTGGCGTTACCTTGG-3', 5'-AGCCCTGTATTCCGTCTCCT-3'; Smad2: 5'-GGGAGCAGAATATCGGAGGC-3', 5'-GCTTGAGCATCGCACTGAAG-3'; β-actin: 5'-CTGTCCCTGTATGCCTCTG-3', 5'-ATGTCACGCACGATTTCC-3'.

Western blotting

Tumor cells were collected from rat ascites and added with 1 mL of cell lysate. After ultrasonic crushing and centrifugation, the supernatant was removed, and 100 μg of protein was loaded per well. Protein samples were separated using SDS-PAGE, wet-transferred at 4°C, 100 V for 1 h, and sealed with 5% skimmed milk for 2 h. Afterwards, the following primary antibodies were added for incubation at 4°C overnight: β-actin (1:1000), TGF-β1 (1:500) and Smad2 (1:500). Then, HRP-conjugated secondary antibody was added (1:1000) and incubated for 2 h in dark. DAB method was used for color development and fixation, and the bands were scanned. The relative expression of the target protein was represented by the ratio of the absorbance of the target band to the β-actin band.

Statistical analyses

The data were processed using SPSS 22.0 statistical software. The measurement data were described by (mean ± SD) and analyzed using t test. The enumeration data were described by n (%) and analyzed using χ^2 test. Graphpad Prism 7 was used to plot the graphs. A statistically significant difference was defined at $P<0.05$.

Results

General condition of rats

Before modeling, all the rats had normal diet and free movement. During the modeling process, rats in MG, LDG, MDG and HDG all showed different degrees of dull fur, reduced food intake, slower activity speed, less energy and increased abdominal circumference. Post-test anatomy showed thickened and adhered greater omentum in MG. Miliary nodules of varying diameters were present in the intestine and peritoneum. The macroscopic observation showed that MDG and HDG had less degree of intestinal adhesion than MG and LDG. This indicated that the experimental model was successfully established, and the medium and

Table 1. Comparison of PLT, WBC and SF ($\bar{x} \pm s$)

Group	Number of cases	PLT (× 10^9/L)	WBC (× 10^9/L)	SF (μg/L)
Control group	10	179.36±19.36	6.69±1.25	61.85±8.76
Model group	10	112.24±15.42***	4.16±0.88***	89.78±10.55***
Low dose group	10	114.31±18.67###	4.22±0.81###	86.64±5.24###
Medium dose group	10	135.84±12.33###	5.08±0.74###	77.39±4.77###
High dose group	10	158.28±10.08###	5.89±0.62###	70.25±3.19###
F	-	34.278	15.135	26.735
P	-	<0.001	<0.001	<0.001

PLT: Platelet; WBC: White Blood Cell; SF: Serum Ferritin. ***P<0.001 compared with the control group; ###P<0.001 compared with the model group.

high doses of Xiaoaiping improved the malignant ascites in rats.

PLT count, WBC count and SF

There were significant differences in PLT count, WBC count and SF among all the groups (P<0.05). The MG showed fewer PLT and WBC counts, and higher SF than the CG (P<0.05). PLT and WBC counts increased sequentially, and SF decreased sequentially in the LDG, MDG and HDG (P<0.05). This indicated that rats with malignant ascites had abnormal PLT, WBC and SF, and Xiaoaiping could improve PLT, WBC and SF in a dose-dependent manner (**Table 1**).

Thymus index, spleen index, kidney index and renal function

The thymus index in the MG was remarkably lower than that in the CG (P<0.05). The thymus index in the LDG, MDG and HDG were remarkably higher than that in the MG (P<0.05). No statistically significant difference was observed in spleen index among all the groups (P>0.05). The kidney index, Scr and BUN in the MG were higher than those in the CG (P<0.05) but noticeably lower than those in the LDG, MDG and HDG (P<0.05) (**Figure 1**).

Peripheral blood T cells

Compared with the CG, the MG showed markedly decreased ratio of CD4+ cells and CD4+/CD8+ of the peripheral blood, while increased ratio of CD8+ (P<0.05). In the LDG, MDG and HDG, the ratio of CD4+ cells and CD4+/CD8+ of the peripheral blood were markedly higher, while the ratio of CD8+ was markedly lower than those in the MG (P<0.05) (**Figure 2**).

Serum cytokines

The serum levels of IFN-γ, IL-1α, IL-1β, IL-2, IL-4 and TNF-α in the MG were markedly higher than those in the CG. Compared with the MG, the MDG showed markedly increased serum levels of above cytokines (P<0.05) (**Figure 3**).

Ascites volume and tumor cell survival rate

The ascites volume and tumor cell survival rate showed noticeable differences among all groups (P<0.05). The volume of ascites and the survival rate of tumor cells in the MG were higher than those in the LDG, MDG and HDG (P<0.05). The volume of ascites and the survival rate of tumor cells were decreased sequentially in the LDG, MDG and HDG (P<0.05). It indicates that Xiaoaiping can inhibit the formation of malignant ascites secondary to gastric cancer or accelerate the excretion of ascites, and inhibit tumor survival (**Table 2**).

mRNA expression of TGF-β1 signaling pathway

Statistically noticeable differences in TGF-β1 and Smad2 were found among all groups (P< 0.05). The MG showed higher mRNA expressions of TGF-β1 and Smad2 than the CG (P<0.05). The mRNA expressions of TGF-β1 and Smad2 in the LDG, MDG and HDG were decreased sequentially (P<0.05). The mRNA expressions of TGF-β1 and Smad2 exhibited no marked differences between the LDG and MG (P>0.05). In contrast to the CG, the differences in the mRNA expressions of TGF-β1 and Smad2 in the HDG were still statistically marked (P<0.05). It showed that Xiaoaiping can regulate the TGF-β1 signal pathway (**Table 3**).

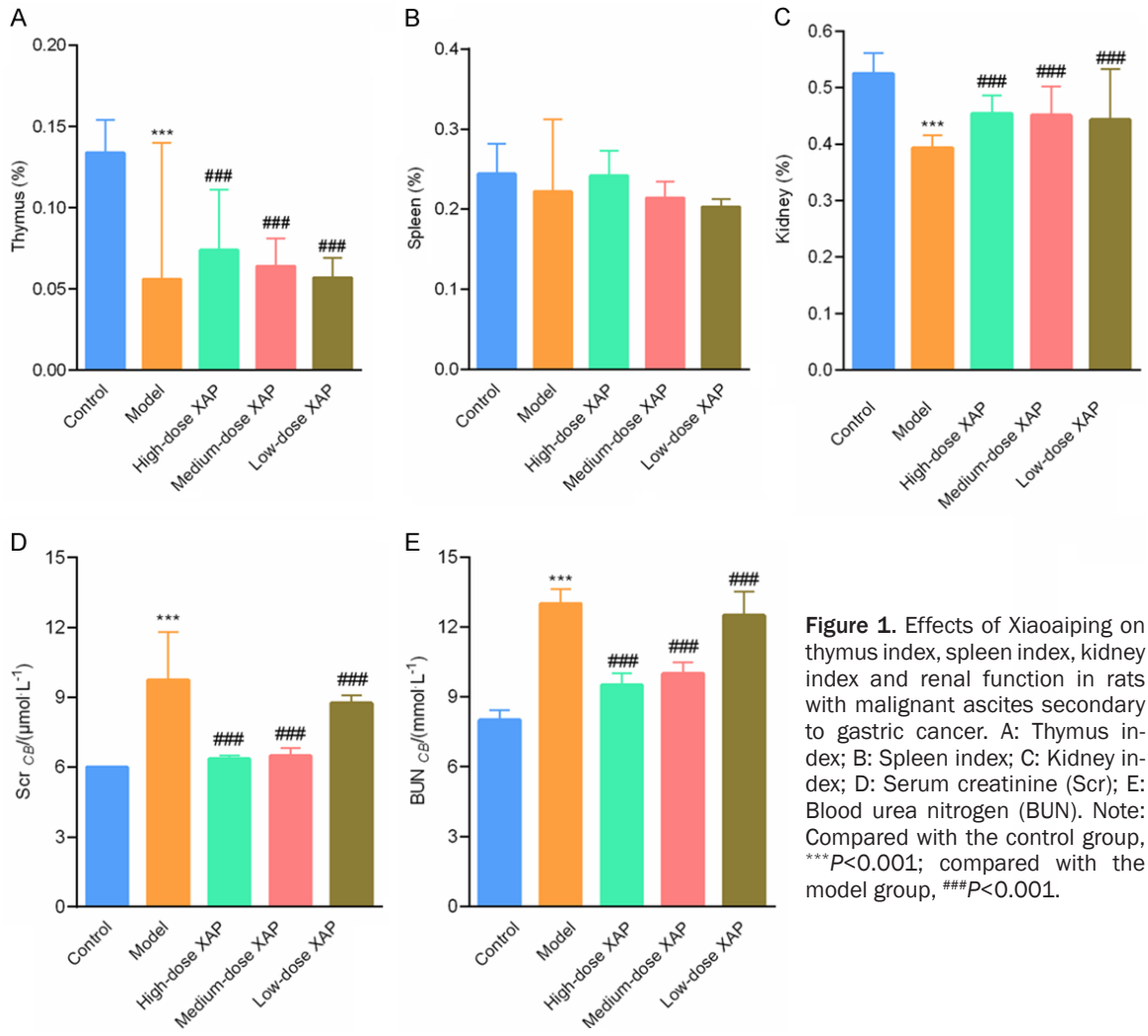

Figure 1. Effects of Xiaoaiping on thymus index, spleen index, kidney index and renal function in rats with malignant ascites secondary to gastric cancer. A: Thymus index; B: Spleen index; C: Kidney index; D: Serum creatinine (Scr); E: Blood urea nitrogen (BUN). Note: Compared with the control group, $^{***}P<0.001$; compared with the model group, $^{###}P<0.001$.

Expression of TGF-β1 signaling pathway-related proteins

The MG showed higher protein expressions of TGF-β1 and Smad2 than the CG ($P<0.05$). The protein levels of TGF-β1 and Smad2 in the LDG, MDG, and HDG were declined sequentially ($P<0.05$). The protein levels of TGF-β1 and Smad2 exhibited no marked differences between the LDG and MG ($P>0.05$). In contrast to the CG, the differences in the protein levels of TGF-β1 and Smad2 in HDG were still statistically marked ($P<0.05$). It suggests that Xiaoaiping can regulate the expression levels of TGF-β1 signaling pathway-related proteins (**Figure 4**).

Discussion

Peritoneal metastasis is the leading cause of death in patients with gastric cancer. Malignant ascites secondary to gastric cancer is an early and common clinical symptom of abdominal metastasis, leading to rapid progression and poor prognosis. Hence, it is of great significance to explore the treatment methods and related mechanisms of malignant ascites secondary to gastric cancer. This study found that there were varying degrees of differences in the food intake, mental state, and activity of the rats before and after modeling. Rats in the MG, LDG, MDG and HDG all had different degrees of dull fur, reduced food intake, slower activity, less energy and increased abdominal circumference compared with the CG, indicating that the experimental model was successfully established. After the test, the greater omentum was thickened and adhered in the MG, and the miliary nodules of varying diameters were present in the intestine and peritoneum. The degree of intestinal adhesion in the MDG and HDG was less than that in the MG

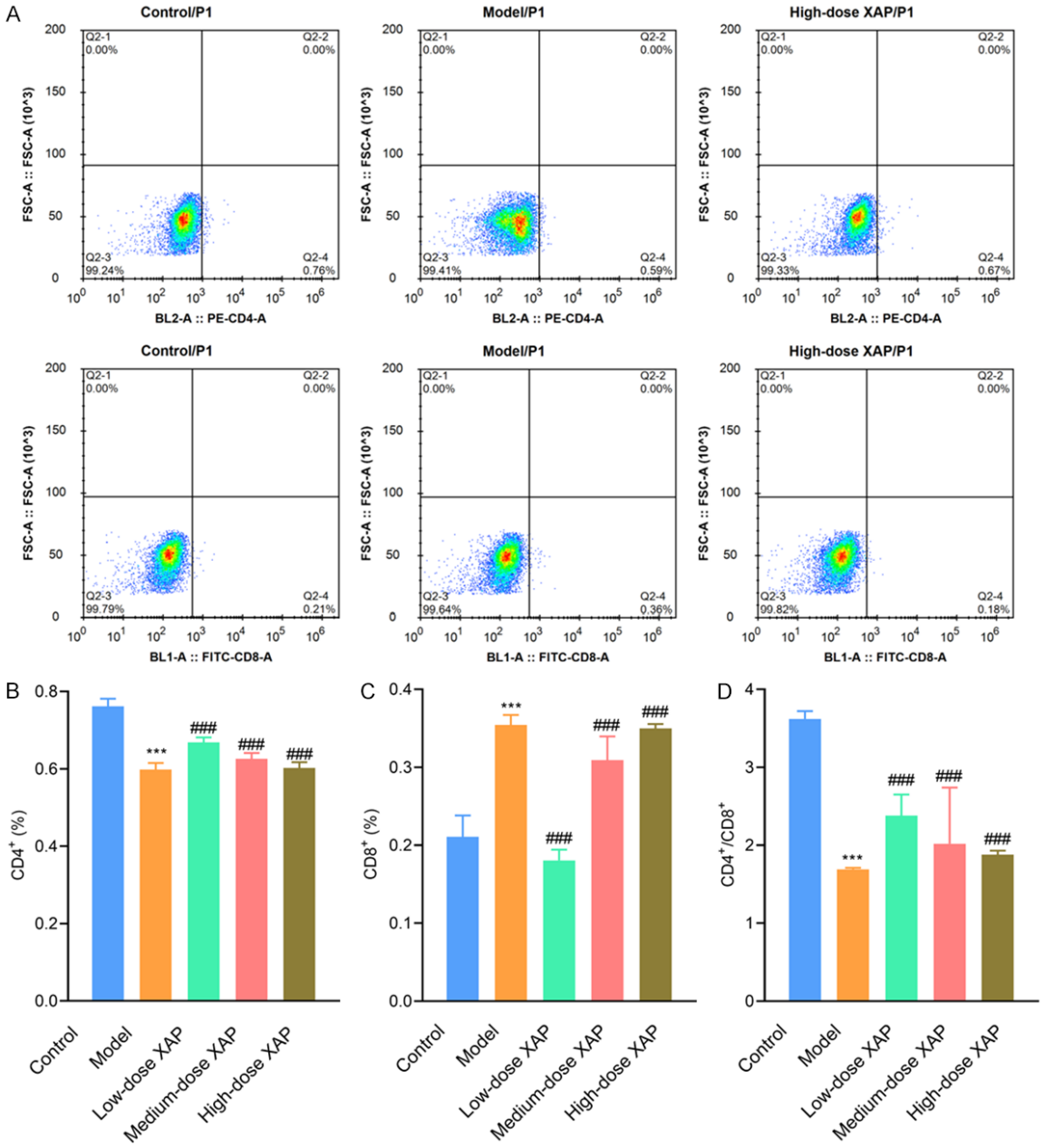

Figure 2. Effect of Xiaoaiping on peripheral blood T lymphocytes in rats with malignant ascites secondary to gastric cancer. A: Flow cytometry images of CD4+ and CD8+ (control, model and high-dose groups); B: CD4+; C: CD8+; D: CD4+/CD8+. Note: Compared with the control group, $^{***}P<0.001$; compared with the model group, $^{###}P<0.001$.

and LDG, indicating that the medium and high doses of Xiaoaiping could improve the condition of malignant ascites secondary to gastric cancer in rats. The MG exhibited higher volume of ascites and survival rate of tumor cells than the LDG, MDG and HDG, and the volume of ascites and the survival rate of tumor cells were decreased sequentially in the LDG, MDG and HDG, indicating that Xiaoaiping could inhibit the formation of malignant ascites secondary to gastric cancer or accelerate the ascites excretion, inhibit tumor survival, and improve the general condition of rats.

In traditional Chinese medicine, it is believed that damp-heat is a crucial pathogenesis of ascites. Malignant ascites secondary to gastric cancer is a long-term consequence of the dis-

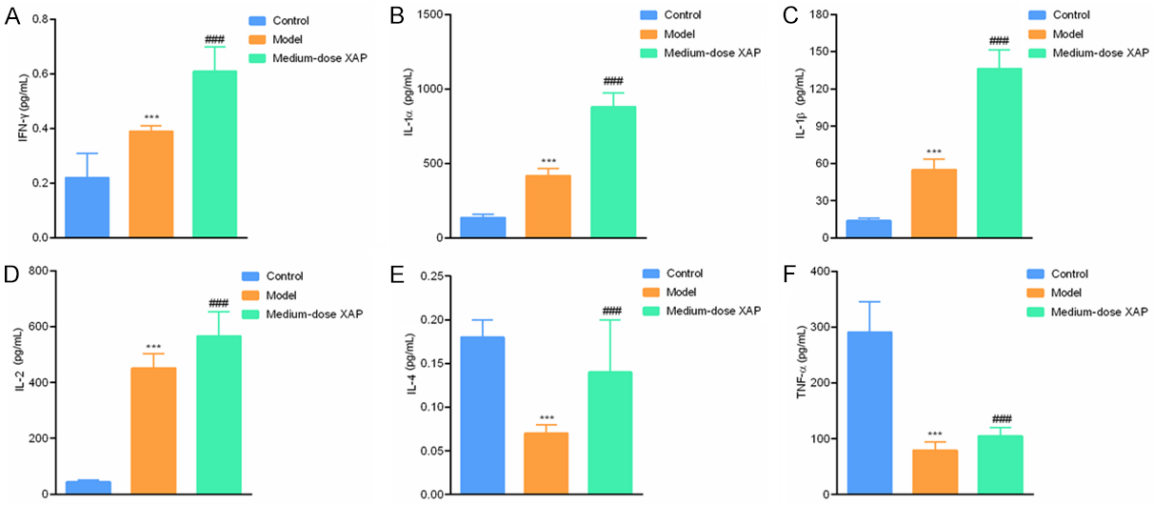

Figure 3. Effects of Xiaoaiping on peripheral serum cytokines in rats with malignant ascites secondary to gastric cancer. A: Interferon γ (IFN-γ); B: Interleukin-1α (IL-1α); C: Interleukin-1β (IL-1β); D: Interleukin-2 (IL-2); E: Interleukin-4 (IL-4); F: Tumor necrosis factor-α (TNF-α). Note: Compared with the control group, $^{***}P<0.001$; compared with the model group, $^{###}P<0.001$.

Table 2. Comparison of ascites volume and tumor cell survival rate (mean ± SD)/(%)

Group	Number of cases	Volume of ascites (mL)	Tumor cell survival rate (%)
Control group	10	0.00±0.00	0.00
Model group	10	10.26±0.54	59
Low dose group	10	9.74±0.61	48
Medium dose group	10	7.02±0.35$^{###}$	42$^{###}$
High dose group	10	4.16±0.44$^{###}$	32$^{###}$
F/χ^2	-	322.149	101.825
P	-	<0.001	<0.001

Note: $^{###}P<0.001$ compared with the model group.

Table 3. Comparison of mRNA of TGF-β1 signaling pathway among groups (mean ± SD)

Group	Number of cases	TGF-β1	Smad2
Control group	10	2.16±0.52	1.11±0.25
Model group	10	10.97±1.36***	5.31±1.46***
Low dose group	10	9.91±1.58	5.16±1.18
Medium dose group	10	6.23±1.19$^{###}$	3.72±0.81$^{###}$
High dose group	10	5.04±1.26$^{###}$	2.31±0.54$^{###}$
F	-	85.291	36.406
P	-	<0.001	<0.001

Note: TGF-β1: Transforming Growth Factor-β1. $^{***}P<0.001$ compared with the control group; $^{###}P<0.001$ compared with the model group.

ease. Stomach heat hurts the Yin. The treatment should clear heat and nourish Yin, benefit the stomach and produce fluid. Xiaoaiping has the functions of clearing heat and detoxifying, resolving phlegm and softening firmness, so it is suitable for the treatment of malignant ascites secondary to gastric cancer.

A previous study suggested that abnormal expressions of cytokines played a crucial part in the process of peritoneal metastasis [11]. TGF-β1 is regarded as one of the most potent stimulators of human peritoneal mesothelial fibrosis [12]. However, there are few reports on whether the effects of Xiaoaiping on malignant ascites secondary to gastric cancer in rats involve the TGF-β1 signaling pathway. This study showed that the MG had higher mRNA expressions of TGF-β1 and Smad2 than the CG ($P<0.05$). After intervention, TGF-β1 and Smad2 mRNA levels were reduced sequentially in the LDG, MDG and HDG ($P<0.05$), suggesting that the TGF-β1 signaling pathway may be one of the targets of Xiaoaiping. Meanwhile, no significant difference was observed in TGF-β1 and Smad2 levels between the LDG and MG ($P>0.05$), which is consistent with the macro-

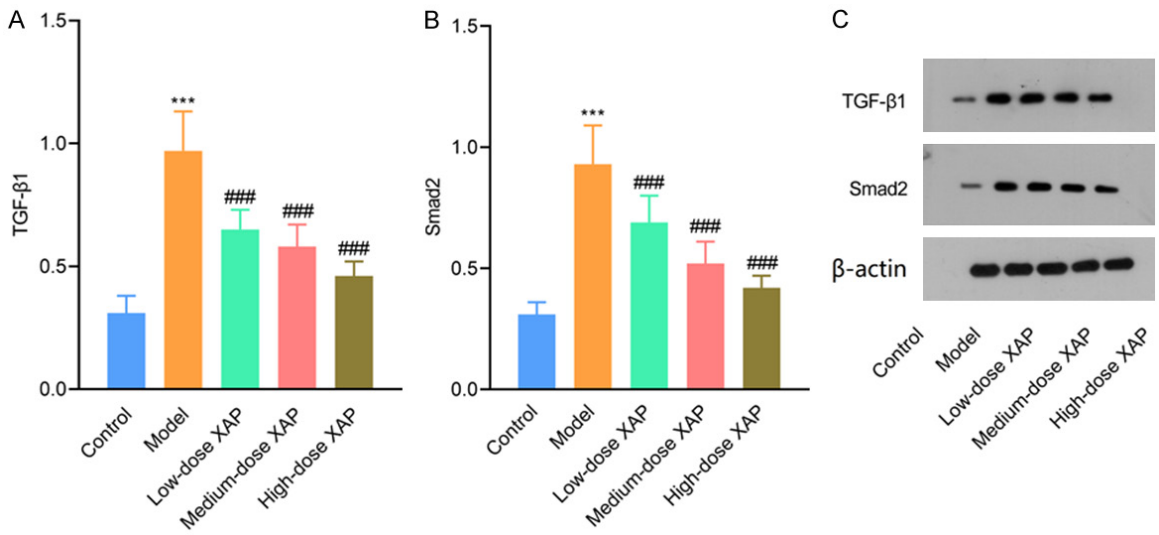

Figure 4. Effects of Xiaoaiping on expressions of TGF-β1 signaling pathway-related proteins. A: Transforming growth factor-β1 (TGF-β1); B: Smad2; C: Western blotting images of protein expressions of TGF-β1 and Smad2. Note: Compared with the control group, $^{***}P<0.001$; compared with the model group, $^{###}P<0.001$.

scopic observation that the degree of intestinal adhesion in the MDG and HDG was slighter than that in the MG and LDG. It is indicated that low dose of Xiaoaiping does not improve the expression of TGF-β1 signaling pathway significantly. It is worth noting that, the TGF-β1 and Smad2 levels were still significantly different between the HDG and the CG, suggesting that Xiaoaiping alone could completely recover the condition of rats with malignant ascites secondary to gastric cancer. First, TGF-β1 binds to the transmembrane glycoprotein TβRII, activates TβRI through phosphorylation, forms a TβRI-TGF-β1-TβRII heterotetramer, and transmits the signal into the cell to rephosphorylate TβRI and activates Smad2 [13-16]. Smad2-activated SH2 region can bind to the phosphorylated tyrosine-binding region and synergistically activate the transcription of target genes with Smad4 [17, 18]. A trial of intermediate and advanced nasopharyngeal carcinoma showed that positive expression of TGF-β1 was associated with tumor metastasis [19]. Zhang et al. [20] established an animal model of gastric cancer and found that the expressions of TGF-β1 and Smad2 were markedly increased in gastric cancer model rats compared with healthy rats.

It has been reported that Xiaoaiping has certain anti-tumor angiogenesis effects [21-23]. In in-vitro experiments, Xiaoaiping injection down-regulated the expressions of phosphorylated vascular endothelial growth factor receptor 2 and phosphorylated protein kinase B protein, thereby regulating angiogenesis and metastasis [24]. In a previous study, the proliferation and invasion ability of gastrointestinal stromal tumor cells were reported to be significantly increased after TGF-β1 activated TGF-β/Smads signaling pathway, and decreased after blocking TGF-β1/Smads signaling pathway [25]. These findings all confirmed that Xiaoaiping had an anti-tumor effect via regulating the TGF-β signaling pathway. It has been found that most patients with malignant ascites secondary to gastric cancer have low immune function, fewer PLT and WBC counts, high SF and poor condition [26-28]. The blood routine test in this study showed that the MG exhibited fewer PLT and WBC counts, and higher SF than the CG. In the Xiaoaiping groups, PLT and WBC counts were increased sequentially, while SF was decreased sequentially, suggesting that Xiaoaiping could improve the condition of rats with malignant ascites secondary to gastric cancer in a dose-dependent manner.

In conclusion, Xiaoaiping can down-regulate TGF-β1 and Smad2 mRNA levels in a dose-dependent manner to inhibit the formation of malignant ascites or accelerate the excretion of ascites, and inhibit the survival of peritoneal tumor cells, thereby improving the condition of rats. Regulation of TGF-β1 signaling pathway may provide reference for the clinical targeted therapy on malignant ascites secondary to gastric cancer.

Disclosure of conflict of interest

None.

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