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ORIGINAL ARTICLE

Basic Study

Effects of invigorating-spleen and anticancer prescription on extracellular signal-regulated kinase/mitogen-activated protein kinase signaling pathway in colon cancer mice model

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Abstract

BACKGROUND

Colon cancer (CC) is one of the most common malignant tumors in the gastrointestinal system. Overall, CC had the third highest incidence but the second highest mortality rate globally in 2020. Nowadays, CC is mainly treated with capecitabine chemotherapy regimen, supplemented by radiotherapy, immunotherapy and targeted therapy, but there are still limitations, so Chinese medicine plays an important role.

AIM

To investigate the effects of invigorating-spleen and anticancer prescription (ISAP) on body weight, tumor inhibition rate and expression levels of proteins in extracellular-signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling pathway in CC mice model.

METHODS

The CC mice model were established and the mice were randomly divided into 5 groups, including the control group, capecitabine group, the low-dose, medium-dose and high-dose groups of ISAP, with 8 mice in each group, respectively. After 2 weeks of intervention, the body weight and tumor inhibition rate of mice were observed, and the expression of *RAS*, *ERK*, phosphorylated *ERK* (*p-ERK*), *C-MYC* and matrix metalloproteinase 2 (MMP2) proteins in the tissues of tumors were

detected.

RESULTS

Compared with the control group, the differences of body weight before and after treatment was much smaller in the groups of ISAP, with the smallest difference in the high-dose group of ISAP, while the capecitabine group had the greatest difference, indicating ISAP had a significant inhibiting effect on the growth of transplanted tumor in mice. The expression of RAS protein was decreased in the low- and medium-dose groups of ISAP, and the change of *p-ERK* was significant in the medium- and high- dose groups of ISAP. MMP2 protein expression was significantly decreased in both the low-dose and medium-dose groups of ISAP. There were no significant changes in *ERK* in the ISAP group compared to the capecitabine group, while RAS, MMP2, and C-MYC protein expression were reduced in the ISAP group. The expression level of C-MYC protein decreased after treated with ISAP, and the decrease was the most significant in the medium-dose group of ISAP.

CONCLUSION

ISAP has a potential inhibiting effect on transplanted tumor in mice, and could maintain the general conditions, physical strength and body weight of mice. The expression levels of *RAS*, *p-ERK*, *MMP2* and *c-myc* were also decreased to a certain extent. By inhibiting the expression of upstream proteins, the expression levels of downstream proteins in ERK/MAPK signaling pathway were significantly decreased. Therefore, it can be concluded that ISAP may exert an anti-tumor effect by blocking the ERK/MAPK signaling pathway and inhibiting the expression of MMP2 and c-myc proteins.

Key Words: Colon cancer; Invigorating-spleen and anticancer formula; Extracellular signal-regulated kinase/mitogen-activated protein kinase signaling pathway; Mice model; C-MYC

Core Tip: The incidence and mortality of intestinal cancer is increasing year by year. Due to the limited therapeutic means and low survival rate of advanced patients, and the easy development of drug resistance to chemotherapy, targeting and immunotherapy, we have found that spleen-healthy anticancer formula can play an anti-tumor role through the extracellular-signal-regulated kinase/mitogen-activated protein kinase signaling pathway and inhibit the expression of matrix metalloproteinase 2 and *c-myc*.

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INTRODUCTION

Colon cancer (CC) is among the most common malignant tumors of the digestive tract, ranking third and second worldwide in 2020 in terms of incidence and mortality, respectively[1]. CC typically arises from epithelial cells that line the lumen of the colon, which renew themselves every five days from a stem cell population located at the base of colonic epithelial cell crypts, and is the consequence of a multistep neoplastic process that extends over several years. Treatment of CC is largely based on the chemotherapeutic drug capecitabine, while there have also been remarkable advances in surgery, immunotherapy, stereotactic radiotherapy and new chemotherapy drugs; however, the incidence rate of and mortality from CC have continued increasing. The rising incidence of CC in younger people is related to dietary patterns, excess body weight, and lifestyle factors[2,3]. Further, drug resistance develops in nearly all patients with CC, leading to a decreased in the therapeutic efficacy of anticancer agents[4]. In addition, the presence of nodal involvement (stage III) predicts for a 60% likelihood of recurrence. Surgical resection is highly effective for early-stage CC, providing cure rates of over 90% and 75% in patients with stage I and II disease, respectively[5]. However, approximately 30% of the patients with CC have distant metastasis at diagnosis, indicating that they are unsuitable for surgical treatment[6,7]. The 5-Fu derivative, capecitabine, is an important agent for treatment of CC at all stages. In traditional Chinese medicine, CC is categorized as "abdominal mass" and "perianal abscess". The basic pathogenesis of CC superficially involves asthenia, while deficiency of vital Qi is the pathogenic characteristic of the whole disease process, with “cancer toxin”, “phlegm and blood stasis”, and “dampness and heat” as relevant pathogenic factors. Invigorating-spleen and anticancer prescription (ISAP) has shown good therapeutic effects against CC[8]. Previous cell experiments have demonstrated that ISAP can block the cell cycle and inhibit tumor cell growth and migration[9]. *In vivo* experiments showed that levels of vascular endothelial growth factor and microvascular density, which are closely related to angiogenesis, are significantly

inhibited in tumor-bearing mice; however, the anti-tumor mechanism underlying these effects of ISAP remains unclear. In this study, we evaluated the effects of different doses of ISAP on body weight and tumor inhibition rate in a CC mouse model, and detected the expression levels of proteins in the extracellular-signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling pathway, to explore the mechanism underlying the effects of ISAP in CC.

MATERIALS AND METHODS

Animals and cells

A total of 40 Kunming mice (4-6 weeks old, 18-20 g) were purchased from Liaoning Changsheng Biotechnology Co., LTD. CT26WT CC cells were purchased from Dalian Meilun Biotechnology Co., LTD.

Reagents and instruments

BCA Kit (Beyotime, China), RAS antibody (Abcam, United Kingdom), ERK monoclonal antibody, pERK monoclonal antibody (Cell Singnaling, United States), matrix metalloproteinase 2 (MMP2) monoclonal antibody (Boster, China), myc antibody (Abcam, United States), Goat anti-rabbit IgG (ZSGB bio, China), β-actin (AbCAM-AbBOT, United States), protein Marker (Fermetas, Germany); Automatic microplate reader (Omega, United States), Electrophoresis apparatus (BIO-RAD, United States), tissue dehydrator (Leica Microsystem, China), microelectronic balance (METTLER TOLEDO, Swiss), low speed centrifuge (Eppendorf, Germany).

Experimental drugs

ISAP is mainly composed of *Heterophylla falsestarwort* root 15 g, *Poria cocos* 15 g, *Atractylodes macrocephala* 15 g, *Prepared licorice* 10 g, dried *Tangerine* 10 g, *Rhizoma Pinellinae Praeparata* 10 g, *Bulbus iphigeniae indicae* 15 g, *Smilax glabra Roxb* 15 g, *Hedyotis diffusa* 15 g, *Fritillaria thunbergii* 15 g, and *Jobstears* seed 30 g and purchased from Outpatient Pharmacy of The First Affiliated Hospital of Liaoning University of Traditional Chinese Medicine. All the drugs were in accordance with the description of China pharmacopoeia 2015. After immersed in warm water for 1 hour, drugs were boiled 3 times for 1 hour each time. Subsequently, the combined decoction was centrifuged to remove the residue of drugs and stored at 4 °C for reserve. Tablets of capecitabine was purchased from Qilu Pharmaceutical Company, China. The specific dosage for mice were converted referring to the coefficient between human and mice.

CC mice model

Cell suspension (cell number: 5×10^6/mL) was prepared from the CT26WT CC cells. A 1 mL syringe was used to suck the cell suspension and subcutaneously injected into the right axilla until a bulge was observed. Finally, palpable ovoid masses under the skin indicated the CC mice model was successfully established. The CC mice were numbered, and randomly divided into 5 groups, including the control group, capecitabine group, low-dose, medium-dose and high-dose groups of ISAP using the table of random numbers. Different interventions were administered for 2 weeks. Grouping and doses were shown in Table 1.

At the end of the second week, mice were euthanatized by cervical dislocation. Subsequently, mice were placed in supine position and their limbs were fixed with big nails. The tumor tissues were collected and then cleaned with 0.9% sodium chloride, followed by weighing and photographing. Moreover, the general condition of mice in each group (including the food intake, skin color and activity) was observed. The changes of body weight in each group were observed before and after interventions. Tumor inhibition rate = (1 - tumor weight in the treatment group/tumor weight in the control group) × 100%.

Western blotting for the expression levels of related proteins in the MAPK/ERK signaling pathway

Appropriate amount of tumor tissues obtained from each group were cut into pieces and put into protein lysate for further homogenization. Then the mixture was sucked by pipette and transferred to centrifugal tube. After centrifuged, the total protein was collected from the supernatant and the protein concentration was determined by a BCA kit. A total of 100 μg proteins were sampled, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto polyvinylidene fluoride (PVDF) membrane and blocked with antibodies. The primary antibodies, including *RAS*, *ERK*, *pERK*, *C-MYC*, and *MMP2*, were added to the PVDF membrane at a dilution ratio of 1:1000 and incubated overnight. Then the secondary antibodies were diluted by tris-buffered saline at a ratio of 1:1000 and added to the membrane. After the incubation at room temperature for 2 hours, chemiluminescence was used for color development. The film was scanned and imaged by a scanner, and the molecular weight and gray values of the target bands in the image were analyzed by an Alpha gel image processing system.

Statistical analysis

SPSS 26.0 was used for statistical analysis of the experimental data. All experimental data were expressed as mean ± SD. One-way ANOVA was used for comparison among groups. Student's *t*-test was used for comparison between groups. $P < 0.05$ was considered statistically significant.

Table 1 Grouping and interventions for mice

Group	Number	Dose, g/kg
Control group	8	/
Low-dose group of ISAP	8	9.5
Medium-dose group of ISAP	8	19
High-dose group of ISAP	8	38
Capecitabine group	8	19

ISAP: Invigorating-spleen and anticancer prescription.

RESULTS

Comparison of the general conditions in groups

Two weeks after intervention, the tumor-bearing mice in the capecitabine group had a poorer mental state, obvious emaciation and dull skin color, with less food intake. Compared with the control group, mice in the low-, medium- and high-dose groups of ISAP all showed better mental state, good activity and no obvious abnormality in skin color, especially in the high-dose group.

Comparison of body weight in groups

There was no significant difference in body weight before modeling among all the groups. After interventions, the body weight in all groups decreased compared with that on day D0 before treatment, especially in the control group and capecitabine group ($P < 0.05$; Table 2). The difference of body weight before and after treatment in the low-, medium- and high-dose groups of ISAP was significantly smaller than that in control group (Table 2).

Comparison of tumor inhibition rates in groups

As shown in Table 3 and Figure 1, different doses of ISAP all showed significant inhibitory effect on the growth of transplanted tumor in mice. The average tumor weight of the high- and medium-dose groups was significantly lower than that of the control group, especially in the high-dose group. There was no significant difference of the average tumor weight between the low-dose group and the control group.

Comparison of the expression levels of RAS, ERK and pERK protein in ERK/MAPK signal pathway in groups

As shown in Figure 2A and B, compared with the control group, the expression levels of RAS protein were significantly decreased in both the low- and medium- dose groups of ISAP, but not in the high-dose group of ISAP. There was no significant change in the protein expression of ERK before and after the low-, medium- and high-dose treatment of ISAP. However, the phosphorylated *ERK* (*p-ERK*) decreased significantly with the increase of the dose of ISAP, especially in the medium- and high-dose groups of ISAP.

Comparison of MMP2 and C-MYC protein expression in groups

As shown in Figure 2C and D, the expression of MMP2 protein was significantly decreased in both the low- and medium-dose groups of ISAP compared to the control group. The protein expressions of c-myc were all significantly decreased after treated with low-, medium- and high- dose ISAP ($P < 0.05$), with the most significant decrease in the medium-dose group of ISAP. There was no statistical difference between the medium- and high-dose groups.

DISCUSSION

Traditional Chinese medicine theory holds that the occurrence of CC is mainly related to the deficiency of vital Qi and the invasion of external pathogens. As written in Jing Yue's Complete Work - Miscellaneous internal diseases - Abdominal mass, most of the patients with splenogastric asthenia and related debilitating disorders have lumps in the abdomen, accompanied by pain or swelling. Further, as written in Lingshu - Nine Needles, pathogenic wind from four seasons and eight directions invading the meridians of the human body result in blood stagnation and stubborn diseases, highlighting the potential for exogenous pathogens in qi and blood pathways inside the human body to cause tumor-related diseases. CC is caused by the combination of internal and external factors, and specifically originates from the deficiency of vital Qi, with invasion of external pathogens another important pathogenic factor. ISAP was developed based on the above etiology and pathogenesis of CC described above, and is mainly composed of a decoction of six noble herbs, supplemented with other traditional Chinese medicines which can dissipate masses, such as *Herba Hedyotidis*, *Scutellariae Barbatae Herba*, *Cremastrae Pseudobulbus*, *Glabrous Greenbrier Rhizome*, *Thunberg Fritillary Bulb*, *Coicis Semen* among others; all these drugs have been demonstrated to have clear anti-tumor effect[10-13]. In addition, clinical studies have shown that a modified decoction of six noble herbs can significantly improve anorexia in patients with malignant tumors after

Table 2 The effect of invigorating-spleen and anticancer prescription on body weight of mice (mean ± SD, g)

Group	D0	D14	D14 - D0
Control group	20.22 ± 0.01	18.15 ± 0.03[a]	2.07 ± 0.03
Low-dose group of ISAP	20.47 ± 0.71	18.89 ± 0.34[a]	1.58 ± 0.83
Medium-dose group of ISAP	20.28 ± 0.02	19.08 ± 0.34[a]	1.20 ± 0.34
High-dose group of ISAP	20.39 ± 0.05	19.51 ± 0.59[a,b,c]	0.87 ± 0.07
Capecitabine group	20.46 ± 0.01	16.29 ± 0.08[a]	4.18 ± 0.06

[a]$P < 0.05$ compared to the control group.
[b]$P < 0.05$ compared to the low-dose group of invigorating-spleen and anticancer prescription (ISAP).
[c]$P < 0.05$ compared to the medium-dose group of ISAP.
ISAP: Invigorating-spleen and anticancer prescription; D0: Day 0; D14: Day 14.

Table 3 The effect of invigorating-spleen and anticancer prescription on tumor inhibition rate of mice

Group	Tumor weight (g; mean ± SD)	Tumor inhibition rate (%)
Control group	1.38 ± 0.12	/
Low-dose group of ISAP	1.22 ± 0.03	0.12 ± 0.02
Medium-dose group of ISAP	1.07 ± 0.34[a]	0.31 ± 0.08[b]
High-dose group of ISAP	0.9 ± 0.08[a]	0.34 ± 0.05[b]
Capecitabine group	0.53 ± 0.13[a]	0.59 ± 0.03[b]

[a]$P < 0.05$ compared to the control group.
[b]$P < 0.05$ compared to the low-dose group of invigorating-spleen and anticancer prescription.
ISAP: Invigorating-spleen and anticancer prescription.

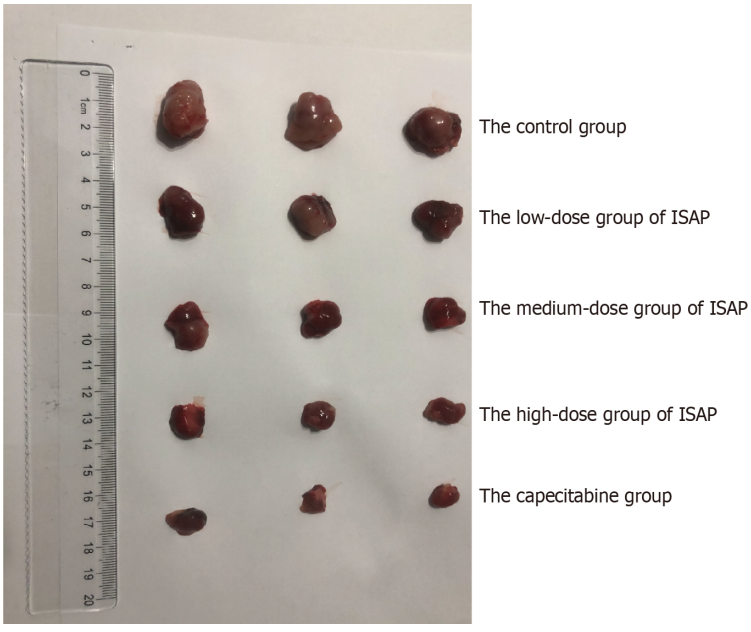

Figure 1 Tissues of transplanted tumors in mice. ISAP: Invigorating-spleen and anticancer prescription.

chemotherapy[14,15], and achieve the effects of invigorating qi, strengthening spleen, anti-cancer and detoxification through the purging-tonifying therapy based on the combinated application of all these drugs. After the spleen and stomach was strengthened, the healthy qi might be restored and the cancerous poison could be exorcised. In this study, results showed that the difference of body weight before and after treatment in different groups of ISAP were much smaller compared to the control group or the capecitabine group, indicating that the body weight of mice was effectively

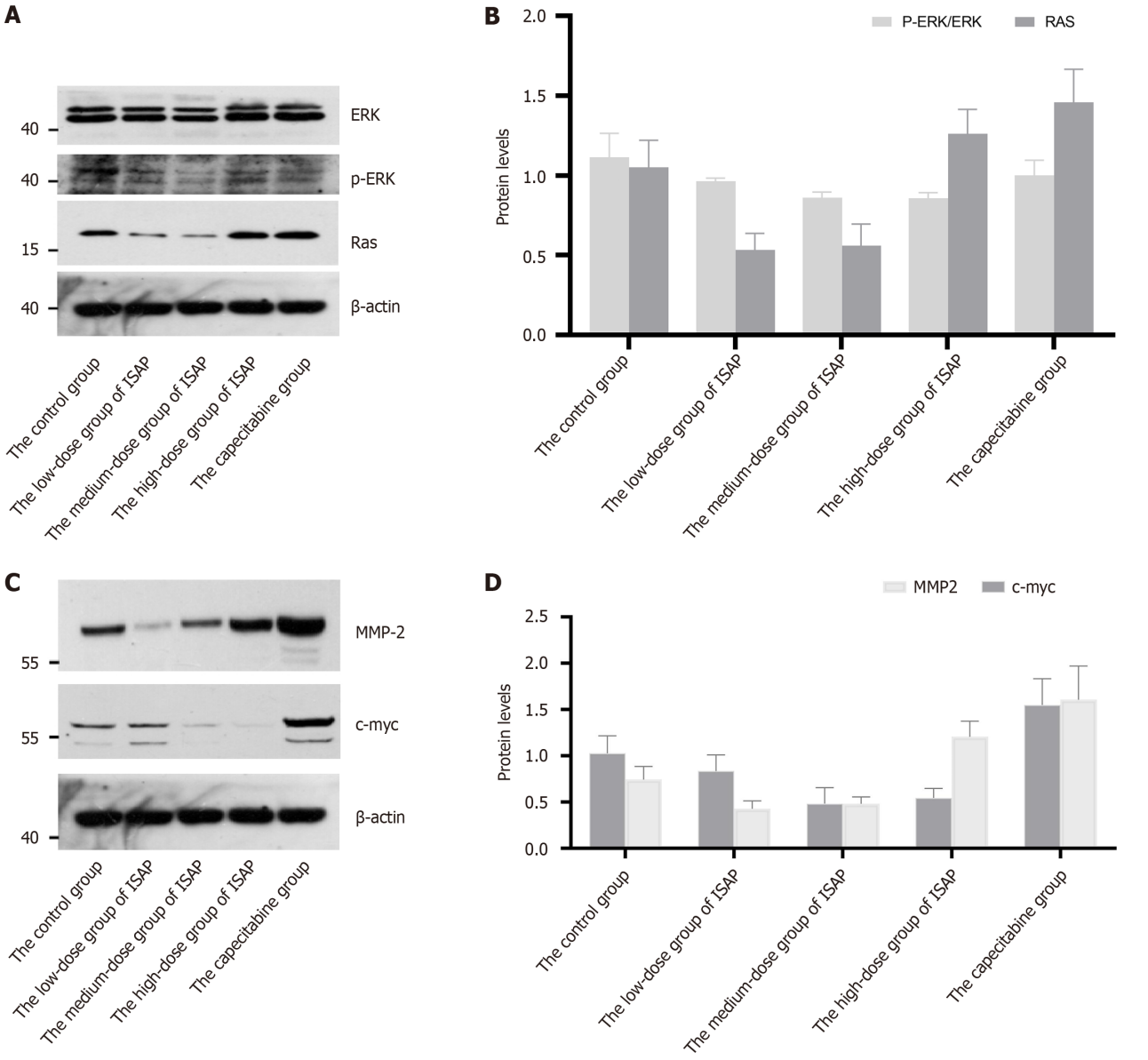

Figure 2 Protein expressions in groups. A: Protein expressions of RAS, extracellular-signal-regulated kinase (ERK) and Perk in groups; B: Comparison of the phosphorylated-ERK/ERK and RAS protein expressions in groups; C: Comparison of matrix metalloproteinase 2 (MMP2) and c-myc protein expressions in groups; D: Comparison of MMP2 and c-myc protein expressions in groups. ISAP: Invigorating-spleen and anticancer prescription; ERK: Extracellular-signal-regulated kinase; p-ERK: Phosphorylated extracellular-signal-regulated kinase; MMP2: Matrix metalloproteinase 2.

stabilized by ISAP, and the general conditions of mice was further maintained. Besides, ISAP had a significant inhibitory effect on the growth of transplanted tumor in mice, and the tumor inhibition rate in the high- and medium-dose groups of ISAP was significantly higher than that in the control group, with the highest in the high dose group, showing a dose-effect relationship to some extent.

MAPK signaling pathway is one of the most widely studied signal transduction pathways, which extensively exists in various human cells. MAPK family proteins in mammals are mainly consisted by *ERK1/2*, *JNK1/2* and *P38 MAPK*; *ERK* mainly has two forms, namely *ERK1* and *ERK2*, and it is activated by the upstream activators of *Ras/Raf* in the sigaling pathway. Together, they constitute the classical Ras-Raf-MAPK signaling pathway, which involves cell proliferation, survival, invasion, migration, apoptosis, glucose metabolism, and DNA repair, are up-regulated oncogenic cascade signals in a variety of tumors[16]. The abnormal activation of Ras-Raf-MAPK signaling pathway promotes cell proliferation, inhibits cell apoptosis, and promote the invasion of tumor tissues to the surrounding or distant areas[17,18]. Studies have demonstrated that MAPK signaling pathway is closely associated with the pathogenesis of colorectal cancer [19], and RAS/MAPK/ERK pathway plays an important regulatory role in cell proliferation and tumor metastasis. *Ras* is involved in transmembrane signal transduction. Activated *Ras* rapidly phosphorylates Raf, turns extracellular signals into specific intracellular signals and links them to nuclear response, thereby amplifying signaling cascade. After binding to *MEK*, *Raf* phosphorylates *MEK*, and phosphorylated *MEK* further phosphorylates *ERK* at two sites (Thr^{188} and Tyr^{190})[20, 21]. Ultimately, *p-ERK* can be transferred from the cytoplasm to the nucleus to activate related transcription factors. In this study, we found that ISAP could inhibit the expression of *RAS* as well as *p-ERK*, both of which were significantly reduced in ISAP group tumors compared with those from control and capecitabine group mice. Expression of the

upstream regulator *RAS*, was not reduced in the high-dose ISAP group, likely due to the greater toxicity effects on this indicator of the drug when used at the high dose, which in turn had an effect on this indicator. However, high and low doses of ISAP did not have a greater effect on the downstream *p-ERK* molecule. We speculate that *ERK* may function synergistically with other molecules. Overall, ISAP can affect CC through the ERK/MAPK signaling pathway.

MMPs are proteolytic enzymes secreted by both tumor cells and normal human cells, among which *MMP-2* and *MMP-9* are the most common subtypes, expressed in the tissues of various malignant tumor and could destroy the histological barriers to facilitate cell invasion. *MMPs* have been found to be closely related to tumor metastasis in liver cancer. As reported by Chen *et al*[22], the high expression of *MMP2* in the tissues of liver cancer could promote the formation of tumor thrombus, which is one of the most reliable indicators of invasion and metastasis of liver cancer. In this experiment, we selected *MMP2* for detection, and found that it was significantly reduced in low and medium dose ISAP groups compared with the control and capecitabine groups, while its levels were not reduced in the high dose ISAP group. We consider that the reasons for these findings may be similar to those discussed above regarding the effects of ISAP on *RAS* levels; that is, they may be due to drug toxicity; however, overall, our data indicate that ISAP can reduce *MMP2* levels.

C-myc is the most ubiquitous member of the myc oncogene family, and participate in the regulation a variety of processes, including the apoptosis, growth and invasion of tumor cells, as well as the angiogenesis and differentiation in tumors[23-26]. A wide variety of naturally occurring tumors exhibit both chromosomal translocations and amplification of the *c-myc* locus that result in constitutive overexpression of myc proteins[27], indicating the inhibition of *c-myc* overexpression could inhibit the occurrence and development of tumors. *C-myc* is also deeply involved in MAPK signaling pathway[28]. The activated ERK is able to activate its downstream substrates, resulting in the changes in gene expression and the levels of various transcription factors[29]. The uncontrolled and abnormal expression of *c-myc* has been found in more than half of the tumors in human. Excessive amplification and abnormal expression of *c-myc* have also been discovered in CC[30].

CONCLUSION

Our experimental data reveal that ISAP can reduce *C-MYC* expression and, taken together, indicated that ISAP may exert antitumor effects by inhibiting ERK/MAPK signaling pathway activation.

FOOTNOTES

Author contributions: Li Z conceived the work, supervised the writing, and provided intellectual input; Wang W and Wang J were responsible for most of the manuscript; All authors conducted the literature search.

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