



RAPID COMMUNICATION

## Inhibitory effect of modified citrus pectin on liver metastases in a mouse colon cancer model

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### Abstract

**AIM:** To discuss the expression of galectin-3 in liver metastasis of colon cancer and its inhibition by modified citrus pectin (MCP) in mice.

**METHODS:** Seventy-five Balb/c mice were randomly divided into negative control group ( $n = 15$ ), positive control group ( $n = 15$ ), low MCP concentration group ( $n = 15$ ), middle MCP concentration group ( $n = 15$ ) and high MCP concentration group ( $n = 15$ ). CT26 colon cancer cells were injected into the subcapsule of mouse spleen in positive control group, low, middle and high MCP concentrations groups, except in negative control, to set up a colon cancer liver metastasis model. The concentration of MCP in drinking water was 0.0%, 0.0%, 1.0%, 2.5% and 5.0% (wt/vol), respectively. Liver metastasis of colon cancer was observed after 3 wk. Enzyme-linked immunosorbent assay (ELISA) was used to detect the concentration of galectin-3 in serum. Expression of galectin-3 in liver metastasis was detected by immunohistochemistry.

**RESULTS:** Except for the negative group, the percentage of liver metastasis in the other 4 groups was 100%, 80%, 73.3% and 60%, respectively. The number of liver metastases in high MCP concentration group was significantly less than that in positive control group ( $P = 0.008$ ). Except for the negative group, the median volume of implanted spleen tumor in the other 4 groups was 1.51 cm<sup>3</sup>, 0.93 cm<sup>3</sup>, 0.77 cm<sup>3</sup> and 0.70 cm<sup>3</sup>, respectively. The volume of implanted tumor in middle and high MCP concentration groups was significantly smaller than that in positive control group ( $P = 0.019$ ;  $P = 0.003$ ). The concentration of serum galectin-3 in positive control

and MCP treatment groups was significantly higher than that in the negative control group. However, there was no significant difference between them. Except for the negative control group, the expression of galectin-3 in liver metastases of the other 4 groups showed no significant difference.

**CONCLUSION:** Expression of galectin-3 increases significantly in liver metastasis of colon cancer, which can be effectively inhibited by MCP.

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**Key words:** Pectin; Colonic neoplasms; Metastasis; Liver; Mice

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### INTRODUCTION

Liver metastasis is the main cause that impacts the therapeutic effect and postoperative prognosis of colorectal cancer. Inhibiting liver metastasis is beneficial to the therapeutic effect and postoperative prognosis of colorectal cancer<sup>[1]</sup>. Galectin-3, a carbohydrate-binding protein on tumor cell surface, is closely related to cell to cell adhesion, aggregation of cancer cells *in vitro*, tumor growth and metastasis *in vivo*<sup>[2,3]</sup>. Galectin-3 is highly expressed in a variety of metastatic cancer cells<sup>[4]</sup>. Galactosyl, a main component of modified citrus pectin (MCP), can specifically inhibit tumor growth and metastasis *in vivo* and galectin-3-mediated functions *in vitro*<sup>[5]</sup>. Few studies are available dealing with the inhibitory effects of MCP on cancer metastasis. The aim of this study was to discuss the inhibitory effect of MCP on liver metastasis in a rat colon cancer model.

### MATERIALS AND METHODS

#### Cell lines

Mouse colon adenocarcinoma cell line (CT-26), preserved

and passaged in our biotechnology laboratory, was cultivated in RPMI-1640 culture medium containing 10% new born calf serum, penicillin G and streptomycin at 37°C in an 5% CO<sub>2</sub> incubator containing 50 mL/L CO<sub>2</sub>.

### Animals

Seventy-five 6-8 wk old Balb/c female mice, offered by Guangdong Medical Laboratory Animal Center (certification No. 2006A019), weighing 20-25 g, were used in this study. The mice were free from specified-pathogens. Experiments were performed in the SPF Animal Laboratory.

### Drugs and reagents

MCP was provided by Centraxinc International, Inc (Francisco, USA). Mouse galectin-3 ELISA kit was provided by R&D Company (Minneapolis, USA). Mouse galectin-3 affinity purified pol was purchased from Jingmei Biotech Co, Ltd (Shanghai, China).

### Main equipments

U.S Beecher tissue microarray meter, ST360 auto ELIASA were purchased from Kehua (Shanghai, China).

### Establishment of mouse model of liver metastasis of colon cancer

Seventy-five Balb/c mice were randomly divided into negative control group, positive control group, low MCP concentration group, middle MCP concentration group and high MCP concentration group. The concentration of MCP in drinking water was 0.0%, 0.0%, 1.0%, 2.5% and 5.0% (wt/vol), respectively. CT26 cells in exponential growth with sufficient NS were used to mix up into a suspension ( $1 \times 10^6$ /mL). The mice were anesthetized with 4% chloral hydrate (10 mL/kg) by injecting into their abdominal cavity and an abdominal wall incision paralleling the left subcostal margin was then made. Laparotomy was performed and 0.05 mL of CT-26 suspension was injected into the spleen. A same volume of NS was injected into the abdominal cavity of mice in the negative control group. The incision was closed with #1 suture. All mice continuously received MCP dissolved in drinking water from the 2nd d after operation, to the necropsy day 21. A same volume of distilled water was given in negative control group. All mice had free access to food and water during the experiment.

### Observation

After a 3-wk observation, the eyeball of mice was removed to collect 0.5-1.0 mL peripheral blood. All mice were killed by decapitation. The abdominal cavity was opened to observe primary neoplasms of the spleen and record the volume and number of neoplasms (volume =  $ab^2/2$ ,  $a$  = max diameter,  $b$  = min diameter). The total volume was recorded if there were more than 2 neoplasms. The number of liver metastases was calculated. All neoplasms were identified with HE staining. Liver metastasis was divided into 4 grades as previously described<sup>[6]</sup>: grade 0: no liver metastases; grade I: 1-5

liver metastases; grade II: 6-10 liver metastases; grade III: more than 10 liver metastases.

### ELISA analysis of galectin-3

Blood sample was centrifuged at 3000 r/min for 5 min to separate serum. The isolated serum was stored at  $\leq -20^\circ\text{C}$ . The serum sample was diluted in a diluent at 1:20. In brief, 100  $\mu\text{L}$  of a diluent was added into each well of a plate and incubated for 2 h at room temperature, and the plate was washed with a washing buffer. One hundred  $\mu\text{L}$  of detection antibody was added into each well of a plate, incubated for 2 h at room temperature, and the plate was washed with a washing buffer. One hundred  $\mu\text{L}$  of a working diluent of streptavidin-HRP was added into each well of a plate, incubated for 20 min at room temperature in the dark, the plate was washed. Finally, 100  $\mu\text{L}$  of a substrate solution was added into each well of a plate, incubated for 20 min at room temperature in the dark, and 50  $\mu\text{L}$  of a stop solution was then added into each well of the plate. A microplate reader was used to read the absorbance at 450 nm, then a standard curve was plotted and a formula was used to fit the OD of standard samples.

### Liver metastasis tissue microarray

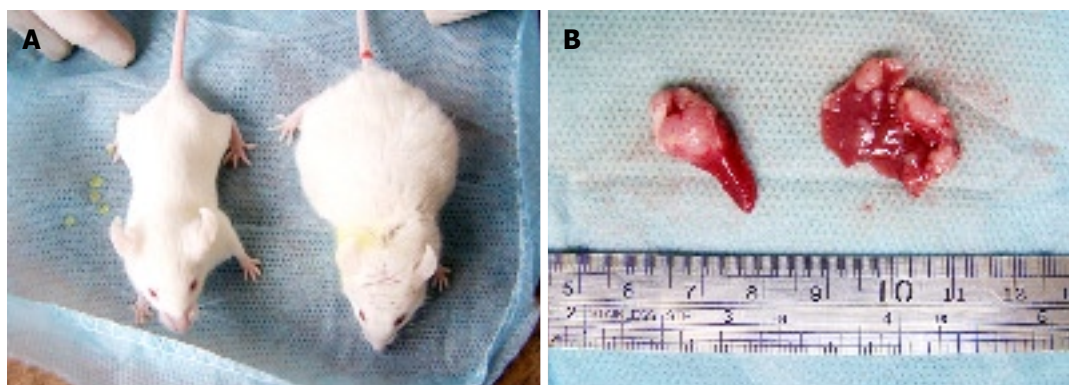
Liver tissue sections were stained with HE to select typical nidi, such as a region rich of neoplasms but lack of necrosed areas and bleeding. A tissue microarray meter was used to perforate into a paraffin block (25 mm  $\times$  25 mm  $\times$  20 mm). The diameter of each hole was 1.2 mm, and the distance between two holes was 1.0 mm. Fifty holes were arranged in 10 lines and 5 arrays. A 1.2-mm long puncture needle was used to draw out the marked typical tissue core and to transfer it to a certain location on the paraffin block. Forty-seven metastasis samples were arranged into 2 paraffin blocks. Each sample included 2 marked cores. The tissue array paraffin was kept on a 55°C copper board for 30 min. The paraffin block was pressed gently to array the tissue cores and cooled at room temperature. The arrays were sliced quickly after pre-cooled at 4°C for 4 h.

### Immunohistochemistry analysis

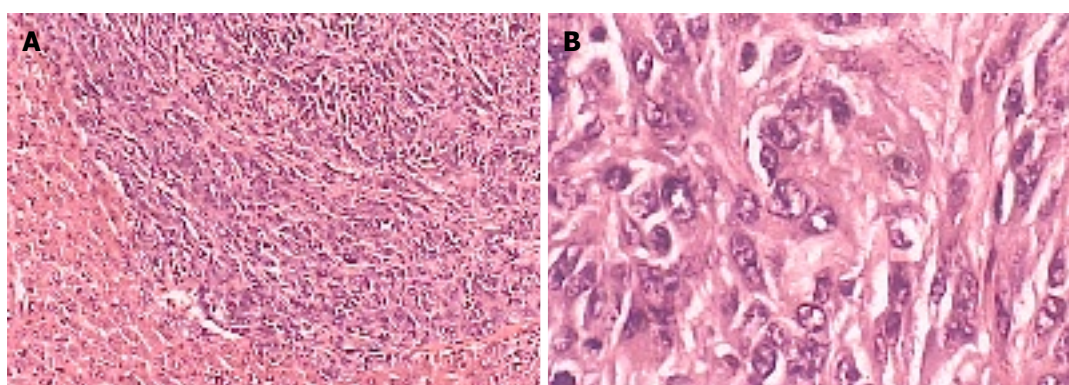
The tissue sample sections were stained with galectin-3 immunohistochemistry following the instructions provided with galectin-3 affinity purified polyclonal antibody. The sections were deparaffined and hydrated. After washed with PBS, the sections were incubated with 3% hydrogen dioxide for 10 min at room temperature, with antibody for 10 min at room temperature, with EnVision for 30 min at room temperature, finally with DAB for color development. The results were judged double-blindly by 2 pathologists. The level of galectin-3 expression was classified into negative (-), weakly positive (+), positive (++) and strong positive (+++) as previously described<sup>[7]</sup>.

### Statistical analysis

All the data were analyzed by SPSS10.0 Software. Tumor



**Figure 1** Mouse model of liver metastatic colon cancer. A: Tumor-bearing and healthy mice; B: Primary spleen tumor and liver metastasis.



**Figure 2** Liver metastatic colon cancer tissue sections stained with HE (A  $\times 200$ , B  $\times 400$ ).

**Table 1** Liver metastases

Groups	n	Numbers of liver metastases				$\chi^2$	P
		0 (0)	I (1-5)	II (6-10)	III (> 10)		
Positive control group	15	0	2	7	6		
1.0% MCP group	15	3	6	2	4	3.996	> 0.05
2.5% MCP group	15	4	7	1	3	7.069	> 0.05
5.0% MCP group	15	6	4	2	3	8.052	< 0.05 <sup>1</sup>

<sup>1</sup>The number of liver metastases in high MCP concentration (5.0%) group was significantly less than that in positive control group ( $P = 0.008$ ).

volume, number of liver metastases, concentration of galectin-3 in serum and tissue were analyzed by non-parametric test.

## RESULTS

### Mouse living status

No mouse died during the 3-wk experiment period. Some mice were found to have tumor mass bulging on the abdominal wall. Some of the cancer-carrying mice appeared signs of mental depression, such as reduced activity, slow response, gloomy hair color, loss of appetite (Figure 1).

### Metastatic liver cancer

Except for the negative control group, the liver metastatic rate for the other 4 groups treated with high, middle and low MCP concentrations was 100%, 80%, 73.3% and 60%, respectively. The number of liver metastases in high

**Table 2** Volume of primary spleen tumor (cm<sup>3</sup>)

Groups	n	M	mean $\pm$ SD	$\chi^2$	P
Positive control group	15	1.51	1.71 $\pm$ 1.29		
1.0% MCP group	15	0.93	1.28 $\pm$ 0.68	2.955	> 0.05
2.5% MCP group	15	0.77	0.90 $\pm$ 0.55	8.083	< 0.05 <sup>1</sup>
5.0% MCP group	15	0.70	0.76 $\pm$ 0.30	7.989	< 0.05 <sup>1</sup>

<sup>1</sup>The volume of primary spleen tumor in middle and high MCP concentration groups was significantly smaller than that in positive control group ( $P = 0.003$ ).

MCP concentration group was significantly less than that in low and middle MCP concentration groups ( $P < 0.05$ ) (Table 1).

### Volume of primary spleen tumor

The median volume of implanted spleen tumor in high, middle and low MCP concentration groups was 1.51 cm<sup>3</sup>, 0.93 cm<sup>3</sup>, 0.77 cm<sup>3</sup> and 0.70 cm<sup>3</sup>, respectively. No tumor was found in negative control group. The volume of tumor in high MCP concentration group was significantly lower than that in middle and low MCP concentration groups ( $P < 0.05$ ) (Table 2, Figure 2).

### Concentration of galectin-3 in serum

The concentration of galectin-3 in serum samples calculated according to the standard regression formula was (14.63  $\pm$  10.08) ng/mL in negative control group, (91.01  $\pm$  22.94) ng/mL in positive control group, (82.75  $\pm$  20.33) ng/mL in low MCP concentration group, (79.01  $\pm$  17.64) ng/mL in middle MCP concentration group and (85.94  $\pm$  15.52) ng/mL in high MCP concentration



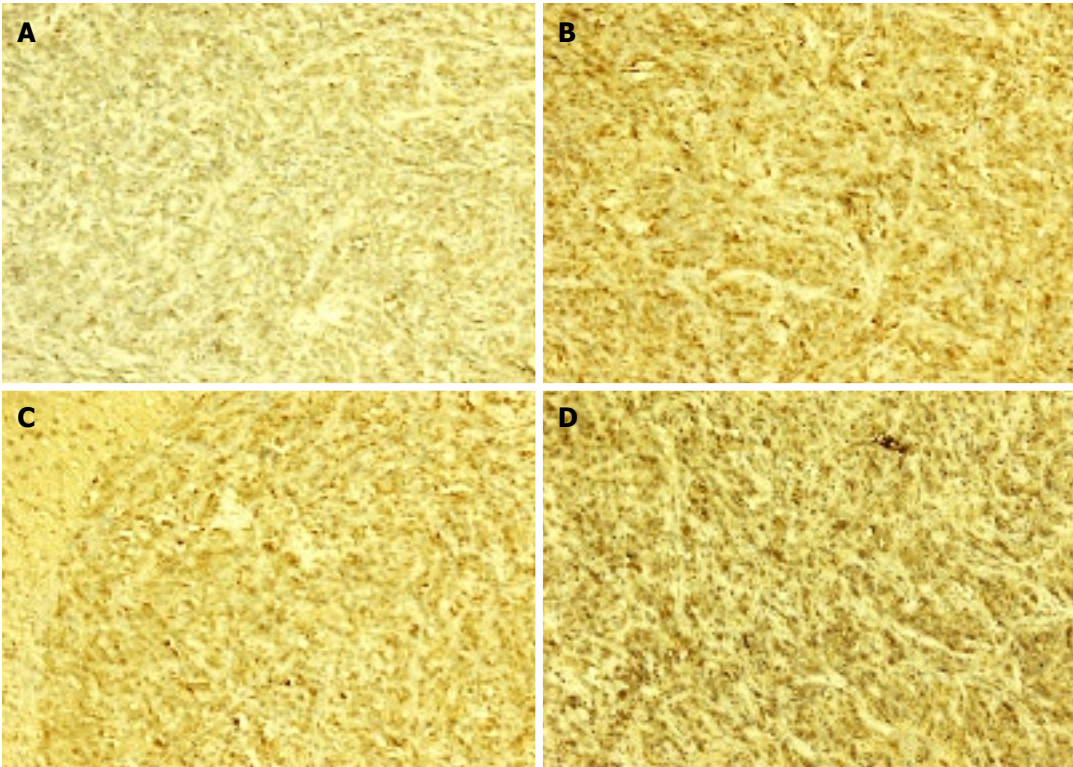


Figure 3 Expression of galectin-3 (× 200) in positive control group (A), 1.0% MCP concentration (B), 2.5% MCP concentration group (C), and 5.0% MCP concentration group (D).

Table 3 Serum concentration of galectin-3 (μg/mL)				
Groups	n	mean ± SD	H	P
Negative control group	15	14.63 ± 10.08	9.37	< 0.01 <sup>1</sup>
Positive control group	15	91.01 ± 12.94		
1.0% MCP group	15	82.75 ± 20.33		
2.5% MCP group	15	79.01 ± 17.64	4.34	
5.0% MCP group	15	85.94 ± 15.52		

<sup>1</sup>The serum concentration of galectin-3 in negatives control group was significantly lower than that in positive control group (*P* < 0.01).

group, respectively. The results indicate that the concentration of serum galectin-3 in positive control group and MCP treatment groups was significantly higher than that in negative control group (*P* < 0.01, Table 3).

Expression of galectin-3 in liver metastasis

Brown cells in cytolymp under microscope were considered positive cells. The percentage of positive cells in metastatic liver tissue showed that galectin-3 had no significant difference in liver metastases positive control and MCP treatment groups (Figure 3, Table 4).

DISCUSSION

Liver metastasis of colon cancer includes tumor cell infiltration, exfoliation, adhesion, aggregation and invasion, which involve carbohydrate-mediated recognition proteins, such as the galectins. Adhesion of tumor cells to tumor embolus and anchorage of tumor cells to blood vessel endothelium or basement membrane are the two crucial steps of liver metastasis of colon cancer. Different galectins expressed in different steps

Table 4 Expression of galectin-3 in liver metastasis							
Groups	n	Expression of galectin-3				H	P
		(-)	(+)	(++)	(+++)		
Positive control group	15	5	6	2	2	0.52	P = 0.170
1.0% MCP group	12	4	3	2	3		
2.5% MCP group	11	2	6	2	1		
5.0% MCP group	9	3	4	1	1		

of metastasis cascade might play a crucial role in tumor progression<sup>[8]</sup>. Galectin-3, a member of the lectin family, is a multifunctional oncogenic protein which regulates cell growth, adhesion<sup>[9]</sup>, proliferation and apoptosis, as well as cell-cell interaction and angiogenesis<sup>[10-13]</sup>. A large body of evidence has confirmed that metastatic cancer cells significantly express galectin-3, and high expression of galectin-3 can be detected in both primary and metastatic lesions<sup>[14]</sup>, even in blood<sup>[15]</sup>, showing a strong relation with cancer growth and metastasis<sup>[16-18]</sup>. Moreover, the expression of galectin-3 can be used as a diagnostic and prognostic marker of colorectal cancer<sup>[19-21]</sup>. Therefore, if the function of galectin-3 is blocked, the progression of adhesion and aggregation can be intercepted, which may stimulate the development of novel drugs for the targeted treatment of colorectal cancer and other cancers<sup>[22]</sup>.

MCP is a non-digestible, water-soluble polysaccharide fiber derived from citrus fruits, and also a complex polysaccharide rich in galactosyl residues. MCP can specifically inhibit carbohydrate-binding protein as a high affinitive ligand<sup>[23]</sup>. When the concentration of MCP reaches an adequate level, galectin-3 protein on the surface of cancer cells would be almost completely blocked by MCP molecules. As a result, the procession of adhesion

and aggregation between cancer cells will be intercepted. In addition, MCP can inhibit morphogenesis of endothelial cells and angiogenesis by blocking galectin-3, thus intercepting cancer cells to absorb nutrition from vessels and cancer progression<sup>[24,25]</sup>. However, there is no evidence that MCP attacks cancer cells directly or indirectly with or without toxicity and side effects<sup>[26]</sup>. *In vitro* experiments have shown that MCP is able to inhibit adhesion of cancer cells to laminin and homotypic aggregation<sup>[27]</sup>. Animal experiments also showed that oral MCP can inhibit the growth and metastases of rat prostate cancer cells<sup>[28]</sup>, human breast cancer<sup>[5]</sup> and melanoma cells<sup>[29-31]</sup>.

The results of our study show that MCP could effectively inhibit the growth and metastasis of implanted colon cancer in mouse spleen. The number of liver metastases and tumor volume in high MCP concentration group were significantly less and smaller than those in control group, indicating that MCP can inhibit the growth and metastasis of colon cancer in a dose-dependent manner, which is consistent with the reported data<sup>[5,28-30]</sup>. In contrast, low MCP concentration group showed no significant difference in colon cancer growth and liver metastasis, which may be due to the lack of samples and the low sensitivity of non-parametric statistics. Further studies are needed to clarify the role of MCP concentration in this regard.

ELISA and immunohistochemistry analysis have shown that MCP does not impact galectin-3 concentration and expression in liver metastatic cancer cells, but inhibits liver metastasis *in vitro*<sup>[30]</sup>. The possible mechanism is that MCP only blocks out galectin-3 molecules on the surface of cancer cells, but does not intercept the expression or secretion of cancer cells. It was recently reported that galectin-3 can be used as a reliable diagnostic marker of colorectal cancer and is one of the target proteins in cancer treatment<sup>[22]</sup>.

In conclusion, MCP can effectively inhibit the growth of colon cancer and liver metastasis by intercepting the adhesion and aggregation of cancer cells. MCP, as a natural polysaccharide derived from fruits and a nontoxic drug, may pave a new way in controlling the growth and metastasis of colon cancer and other cancers. The role of MCP and chemotherapy in controlling and curing liver metastatic colon cancer needs further study.

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## COMMENTS

### Background

Galectin-3 is a carbohydrate-binding protein closely related with cancer growth and metastasis. Studies have shown that galectin-3 is over-expressed in different types of cancer. Dietary components play an important role in cancer progression and metastasis, carbohydrate-mediated recognition processes participate in cancer progression. Modified citrus pectin (MCP), a non-digestible and water-soluble polysaccharide fiber derived from citrus fruits, can inhibit galectin-3-mediated function *in vivo* and *in vitro*.

### Research frontiers

The role of galectin-3 in cancer growth and metastasis is an important field in tumor research. Several experimental studies have reported the specific inhibitory effect of MCP on different cancer cells in xenograft models. The present study investigated the effect of MCP on colon cancer growth and liver metastasis *in vivo*. In addition, the expression of galectin-3 was also studied to describe the possible anticancer mechanism of MCP.

### Innovations and breakthroughs

Few experiments have been conducted to observe the effect of MCP on preventing cancer growth and metastasis *in vivo*. This study showed the inhibitory effect of MCP on liver metastasis of colon cancer in a mouse model. Besides, the expression of galectin-3 in tumor tissue and serum was tested to describe the galectin-3 status during MCP treatment.

### Applications

This study demonstrated that galectin-3 was over-expressed in liver metastasis of colon cancer and oral MCP could inhibit cancer growth and metastasis in a mouse model. The results show that galectin-3, as a potential marker and therapeutic target of colorectal cancer, played an important role in prevention and treatment of cancer.

### Terminology

Citrus pectin is a complex polysaccharide fiber derived from the pulp and peel of citrus fruits. Citrus pectin is rich in galactosyl, a ligand for galectin-3, when it is modified by high-pH and temperature. MCP inhibits galectin-3 function when citrus fruits are combined with galectin-3.

### Peer review

In this manuscript, the authors studied the inhibitory effect of MCP on liver metastasis in a mouse colon cancer model. The study demonstrated that MCP inhibited liver metastasis by suppressing the function of galectin-3. The study is well designed and the results are reliable.

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