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ORIGINAL ARTICLE

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The MMP-9 expression determined the efficacy of postoperative adjuvant chemotherapy using oral fluoropyrimidines in stage II or III colorectal cancer

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Abstract *Background*: The aim of this study was to determine any correlation between the efficacy of postoperative adjuvant chemotherapy using oral fluoropyrimidines and the matrix metalloproteinase 9 (MMP-9) expression in primary colorectal cancer tissues. *Patients and Methods*: The data on 307 patients with colorectal cancer at stage II or III, who underwent potentially curative resection with lymphadenectomy, were reviewed. Of these, 188 received postoperative administration of oral fluoropyrimidines such as UFT and 5′-DFUR (chemotherapy group), while the other 119 patients underwent surgery alone (surgery-alone group). Immunostaining for MMP-9 was performed using surgical specimens of all 307 primary tumors and 18 recurrent tumors. *Results*: Overall, MMP-9 was positively expressed in the primary tumor in 44% of patients. Multivariate analysis revealed that the MMP-9 expression was a worse prognostic factor with a second highest hazard ratio for recurrence. The disease-free survival rate in the chemotherapy group was significantly higher than that in the surgery-alone group. However, no significant difference in disease-free survival rate between the two groups was found in patients with a tumor positive for MMP-9. There was a strong positive correlation of MMP-9 expression between the primary tumors and the recurrent liver or lung tumors. *Conclusions*: The efficacy of postoperative adjuvant chemotherapy using oral fluoropyrimidines such as UFT and 5′-DFUR may not be as great for patients with a tumor positive for MMP-9 having a greater risk to postoperative recurrence.

Keywords MMP-9 expression · Immunohistochemistry · Postoperative adjuvant chemotherapy · Oral fluoropyrimidine · Colorectal carcinoma · Molecular targeting therapy for MMP

Introduction

Colorectal cancer remains one of the leading causes of death in the world. The mainstay for treatment of colorectal cancer with curative intent is surgical resection. In node-positive or stage III patients, however, surgery-alone offers curability to only about 50% of patients treated [1]. Thus, addressing the high risk of recurrence necessarily involves the use of chemotherapy, immunotherapy, or molecular targeted therapy after surgical removal of the primary lesion.

Major advances in adjuvant therapy for colorectal cancer have been achieved since the 1980s. Worldwide, infusion of 5-fluorouracil (5-FU) plus leucovorin (LV) combination chemotherapy (5-FU/LV) has been considered to be standard for stage III colorectal cancer for the past decade [2]. And recently, FOLFOX4 which involves oxaliplatin added to a bolus plus infusion 5-FU/LV has been reported to be more effective than 5-FU/LV in stage II or III colon cancer [3]. In Japan, since the 1980s, oral 5-FU derivatives (fluoropyrimidines) such as UFT (1:tegafur + 4:uracil) and 5′-deoxy-5-fluorouridine (5′-DFUR), an intermediate of capecitabine (N^4 -pentyloxycarbonyl-5-deoxy-5-fluorocytidine), have been used as postoperative adjuvant chemotherapy for colorectal cancer. A recent meta-analysis has reported that surgery combined with the oral fluoropyrimidines was more effective in patients with colorectal cancer at stage II or III than treatment by surgery alone [4, 5].

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It has been shown that the postoperative adjuvant chemotherapy using capecitabine or UFT/oral LV is not inferior to bolus 5-FU/LV [6, 7]. However, the relative efficacy of postoperative adjuvant chemotherapy using oral fluoropyrimidines such as UFT without oral LV or 5′-DFUR compared to intravenous 5-FU/LV so far remains unclear. It is therefore important to know which patients do not respond to oral fluoropyrimidines such as UFT and 5'-DFUR, in order to employ more intensive chemotherapy or some effective molecular targeting therapy.

We have previously investigated the worse prognostic factor of matrix metalloproteinase (MMP) −9 expression in colorectal cancer [8] and have found synthetic MMP inhibitor MMI270 (Novartis Pharma AG, Basel, Switzland) to be effective against postoperative lung metastasis through inhibition of tumor neovasculalization in orthotopic implanted colon cancer in nude rat (Ogata et al.). However, our preclinical study also indicated that early inhibition in tumor neovasculalization might be essential for the efficacy of MMI270. Thus, reinforcing the antitumor effect of MMI270 may be necessary through some combination with conventional cytotoxic drugs as an adjuvant therapy after curative surgery in colon cancer if the neovascular network has already become developed as is usually seen in clinical situations. In the present study, we have investigated the correlation, if any, between the efficacy of adjuvant chemotherapy using oral fluoropyrimidines and the MMP-9 immunoexpression in colorectal cancers, and discuss the application of molecular targeting therapy for MMP as postoperative adjuvant therapy in combination with oral fluoropyrimidines.

Materials and methods

Patients and postoperative adjuvant chemotherapy

Among consecutive 387 patients with a pathological stage II or III colorectal cancer according to the UICC classification of colorectal carcinomas who underwent potentially curative resection with lymphadenectomy including the mesenteric lymph nodes at Kurume University Hospital between 1990 and 1998, 307 patients were enrolled in this retrospective study. When the distal margin of clinical stage II or III rectal cancer was located below the peritoneal reflection, then preoperative adjuvant radiotherapy was indicated, in our institute, and such patients were excluded from this study. The other exclusion criteria were preoperative chemotherapy, any immunotherapy, any radiotherapy, and postoperative chemotherapy except oral fluoropyrimidines. In the enrolled patients with a low rectal tumor in which the distal margin was located below the peritoneal reflection, resection of the pelvic lymph node belonging to the iliac artery was performed in addition to total mesorectal excision. Postoperative adjuvant chemotherapy using oral fluoropyrimidines was performed in 188 patients (chemotherapy group). The other 119 patients underwent surgery alone (surgery-alone group). Fluoropyrimidine such as UFT and 5'-DFUR was administered perorally for 1 year unless postoperative recurrence occurred. The chemotherapy started from 2 to 4 weeks after the surgery. The usual dosages were 500 mg/m^2/day 5'-DFUR, and 250–300 mg /m^2 /day UFT. The two main reasons for surgery-alone were old age equal to or higher than 75 years and patient's choice.

Follow-up

Follow-up investigations were performed through outpatient visits, letter, and telephone, and the most recent date of contact for each patient was regarded as the final date of confirmation. The follow-up was performed up to the last day of December 2003. The median follow-up period was 64 months in the surgery-alone group, and 87 months in the chemotherapy group. The presence or absence of any recurrence was determined according to our follow-up protocol consisting of physical examination including digital examination every 2–3 months, measurement of serum tumor marker (carcinoembryonic antigen, CEA) level every 2–3 months, and/or by findings on barium enema or colonoscopy every 1–2 years, chest radiography every 6 months, and abdominal ultrasound (US), abdominal computed tomography (CT) or abdominal magnetic resonance imaging (MRI) every 6 months up to 5 years, and according to a modified protocol case-by-case thereafter.

Immunohistochemistry

After an initial review of all available hematoxylin and eosin-stained (HE) slides of the surgical specimens consisting of 307 primary tumors, 12 hepatic recurrences and 6 pulmonary recurrences, we selected two paraffin blocks in which the tumor invasive front edge and the viable tumor were clearly revealed, from each case, for our study. Serial 4 micron thin sections were recut from each block. One section from each block was stained by HE again, a second was immunostained for MMP-9. Immunostaining was performed using the avidin-biotin peroxidase complex method. Anti-human MMP-9 rabbit polyclonal IgG [9] was used as the primary antibody. The deparaffinized thin sections were incubated with 0.3% (v/v) of H_2O_2/methanol for 20 min for blocking of endogenous peroxidase activity. After washing with phosphate buffered saline (PBS), non-specific binding was blocked by incubating the sections with normal animal serum for 30 min. The sections were incubated with the primary antibody overnight at 4°C. After washing, the sections were further incubated with biotinylated second antibody for 30 min at room temperature. The primary antibody was detected using avidin-biotin

peroxidase complex (Vector Laboratories, CA, USA) and 3,3′-diaminobenzidine tetrahydrochloride as the chromogen. These thin sections were also counterstained with methylene blue and mounted. For negative control, the sections were incubated with non-immune IgG in place of the primary antibody.

Statistical analysis

The disease-free survival rates prepared by the Kaplan-Meier method were compared between the chemotherapy group and the surgery-alone group. Multivariate analysis for the factors related to disease-free survival was also carried out using Cox's proportional hazard model. All data were compiled and analyzed by Statistical Analysis Software (SAS) version 6.12, (SAS Institute, Cary, NC, USA). The statistically significant difference in clinicopathological characteristics between the two groups was assessed using the chi-square test, Fisher's exact test, and Student's t test. Differences between the groups in Kaplan-Meier plots were evaluated using the log-rank test.

Results

Immunoexpression of MMP-9 in colorectal cancer tissues

MMP-9 was expressed mainly in the cytoplasm of cancer cells (Fig. 1), and of a few neutrophilic leucocytes, monocytes-macrophages, and fibroblast cells. A patient with positive staining for MMP-9 in more than 10% of all tumor cells was defined as a positive case [8]. Forty four percent of all primary colorectal tumors were positive for MMP-9 expression.

Relationship between the MMP-9 expression and the clinicopathological factors

The correlations between the MMP-9 expression and the clinicopathological factors are shown in Table 1. A positive MMP-9 expression was well correlated with lymph node metastasis, the grade of lymphatic invasion [10], and with the pathological stage. Namely, there was a higher rate of positive MMP-9 expression in tumors associated with metastasis to the mesenteric lymph node or lymph node belonging to the internal iliac artery, and in tumors with severe lymphatic invasion (ly2–3).

Correlation between the MMP-9 expression in primary tumor cells and metastatic tumor cells

Among 18 patients with recurrence to the liver or the lung which could be resected postoperatively, MMP-9 was positively expressed by the cancer cells in 12 primary tumors and in 13 metastatic tumors. In 11 of 12 patients with primary tumors positive for MMP-9, recurrent tumor cells also were positive for MMP-9. There was a positive correlation of MMP-9 expression between the primary tumors and the recurrent tumors ($P = 0.009$) (Table 2).

Disease-free survival

The background of the chemotherapy group and the surgery-alone group is summarized in Table 3. The average age in the surgery-alone group was significantly higher than that in the chemotherapy group. However, there was no difference in sex, distribution of tumor stage, or in tumor location, between the two groups. The rate of recurrence was 31% in the surgery-alone group, and 21% in the chemotherapy group. In both groups,

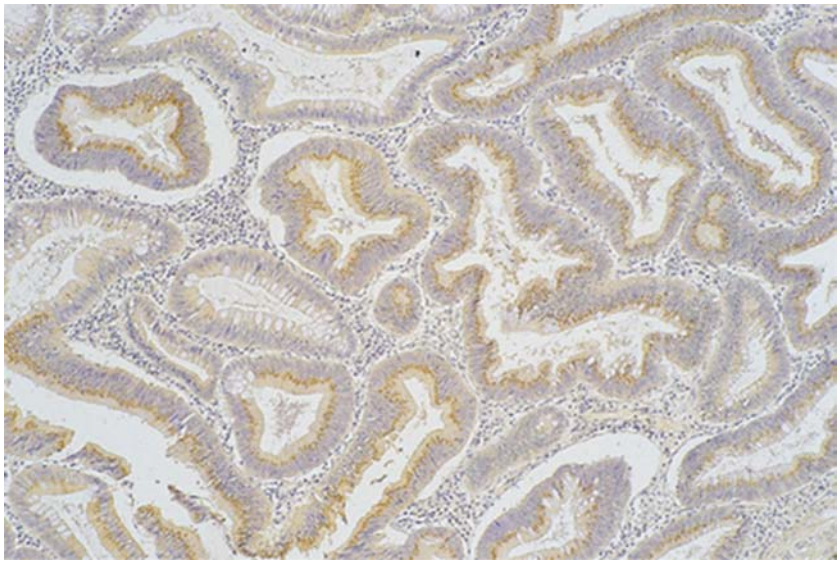

Fig. 1 Immunostaining for MMP-9 in colorectal cancerous tissue. MMP-9 was observed in the cytoplasm of the tumor cells as supranuclear staining. (original magnification; ×100)

Table 1 MMP-9 expression and clinicopathological factors

Factor	MMP-9 expression		*P*-value
	Positive cases/ all cases (%)		
Sex			0.846
Male	77/177	(44)	
Female	58/130	(45)	
Location			0.444
Colon	80/176	(45)	
Rectum	55/131	(42)	
Lymph node metastasis			0.014
MLN (−)	104/255	(41)	
MLN (+)	31/52	(60)	
Lymphatic invasion			< 0.001
ly0-1	84/224	(38)	
ly2-3	51/83	(61)	
Pathological stage			< 0.001
StageII(N0)	44/142	(31)	
StageIII(N1-2)	91/165	(55)	

Lymphatic invasion was defined according to the criteria of Shirouzu [10]
MLN (−): no lymph node metastasis or metastasis to the paracolic (rectal) lymph nodes
MLN (+): metastasis to the mesenteric or the internal iliac lymph nodes

Table 2 Correlation of MMP-9 Expression between the primary tumors and the recurrent tumors

	MMP-9 expression in the recurrent tumors		
	Negative	Positive	Total
MMP-9 expression in the primary tumors			
Negative	4	2	6
Positive	1	11	12
Total	5	13	18

$P = 0.009$ (chi-square value was 6.785)

the major mode of recurrence was hematogenous metastasis, and no difference in the pattern of recurrence was found between the two groups (data not shown). The disease-free survival rate in the chemotherapy group was significantly ($P = 0.01$) higher than that in the surgery-alone group (Figure 2).

The disease-free survival rate in patients with a tumor positive for MMP-9 was significantly lower than that in those negative for MMP-9 (Fig. 3). When the disease-free survival rates were stratified according to the MMP-9 immunoexpression, no significant difference ($P = 0.167$) was found between the chemotherapy group and the surgery-alone group in patients with a tumor positive for MMP-9, whereas significant difference ($P = 0.021$) was demonstrated between the two groups in patients with a tumor negative for MMP-9 (Fig. 4).

Multivariate analysis for disease-free survival rate

Various factors such as postoperative adjuvant chemotherapy, tumor location (colon vs. rectum), histological tumor grade (well vs. others), tumor infiltration (T4 vs. others), lymph node metastasis (− vs. +), metastasis to the mesenteric lymph node or lymph node belonging to the internal iliac artery (MLN− vs. MLN+), grade of lymphatic invasion (ly0–1 vs. ly2-3) and venous invasion (v0-1 vs. v2-3) [11], age, sex, and MMP-9 expression were each evaluated for their independent contributions to the disease-free survival rate after operation using Cox's proportional hazard model. The analysis indicated that surgery-alone and positive MMP-9 expression were significant factors for a worse disease-free survival, in addition to the MLN+ and the severe venous invasion (v2-3) (Table 4).

Discussion

Since the 1990s, intravenous 5-FU/LV therapy has been introduced worldwide as the standard postoperative adjuvant chemotherapy for colorectal cancer [2]. In particular, a significant survival benefit has been established in those who received chemotherapy after curative resection for node-positive or stage III colon cancer, whereas the current evidence does not support the use of adjuvant chemotherapy for all patients with stage II disease [12]. In general, chemotherapy using oral fluoropyrimidines, when comparing to that using intravenous 5-FU/LV, irinotecan [13], oxaliplatin [14], or their combination [15, 16], has been characterized by a lower incidence of adverse effects, especially infrequent adverse effects of grade 3 or higher. This is the reason that the chemotherapy using oral fluoropyrimidines can be continued on an outpatient basis without detriment to the patients' quality of life. For patients, these are critical benefits of the therapy using oral fluoropyrimidines. Recently, a new oral 5-FU derivative capecitabine, which has similar antitumor efficacy to intravenous 5-FU/LV for advanced colorectal cancer [17], has been

Table 3 Background of the patients

	Age* (mean ± sd)	Sex (M/F)	Stage (II/III)	Location (R/C)	MMP-9 expression (positive/negative)	Median follow-up (months)
Surgery alone ($n = 119$)	70.9 ± 10.8	63/56	62/57	52/67	53/66	64
Chemotherapy ($n = 188$)	61.9 ± 10.5	114/74	80/108	79/109	82/106	87

R rectum, *C* colon
*; $P < 0.001$, *M* male, *F* female, *Stage* pathological stage according to the UICC criteria

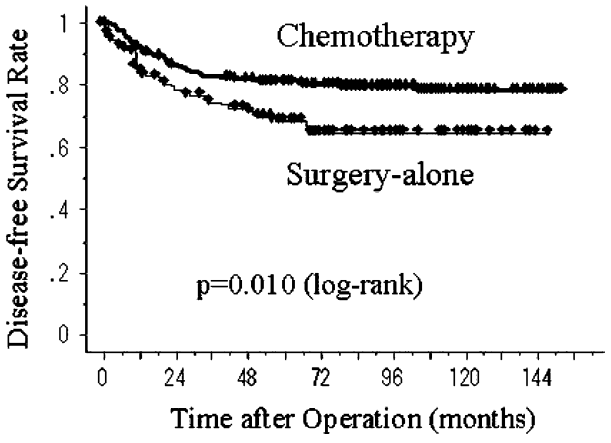

Fig. 2 The disease-free survival curves according to the adjuvant chemotherapy. The disease-free survival rate in the chemotherapy group ($n = 188$) was significantly ($P = 0.010$) higher than that in the surgery-alone group ($n = 119$)

investigated to determine whether its efficacy in postoperative adjuvant therapy for colorectal cancer [18].

A recent meta-analysis has reported that oral fluoropyrimidines such as UFT without oral LV and 5'-DFUR were more effective in preventing recurrence in patients with colorectal cancer at stage II or III than treatment with surgery alone [4, 5]. In our retrospective study, the comparison of disease-free survival rate and the multivariate analysis supported the efficacy of postoperative adjuvant chemotherapy using oral fluoropyrimidines for overall stages II and III colorectal cancers. With regard to the impact of oral fluoropyrimidines, it has been shown that the antitumor efficacy from adjuvant chemotherapy using capecitabine or UFT/oral LV is not inferior to that from intravenous 5-FU/LV [6, 7]. On the other hand, in stage III patients, intravenous 5-FU based regimens have consistently been shown to reduce the risks of recurrence and death by up to 30% [2, 19, 20], which may represent larger benefits than the reduction of 11% in the risk of recurrence and 5% in the risk of death found for oral fluoropyrimidine regimens using UFT without oral LV or 5′-DFUR [4]. It is therefore important to know which patients do not respond to UFT or 5′-DFUR, in order to employ more intensive chemotherapy or some effective molecular targeting therapy.

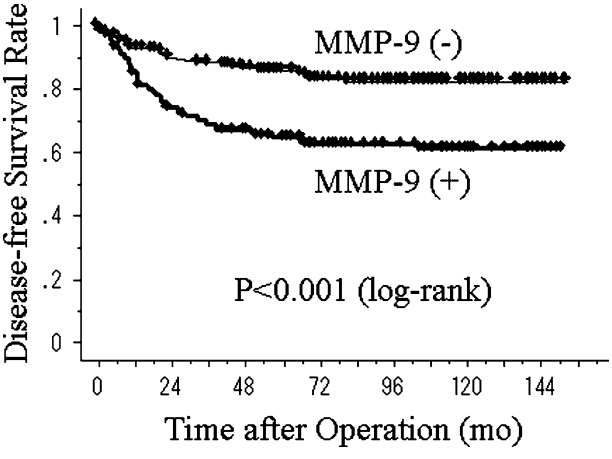

Fig. 3 The disease-free survival curves according to the status of MMP-9 expression. The disease-free survival rate in patients with a tumor positive for MMP-9 ($n = 135$) was significantly ($P < 0.001$) lower than that in those negative for MMP-9 ($n = 172$)

Of note, in biologically aggressive colorectal carcinomas, some biological molecules, such as epidermal growth factor (EGF) [21], vascular endothelial growth factor (VEGF) [22] and MMP, are attractive as a target for cancer treatment. Clinical application of such molecular targeting therapy alone or in combination with chemotherapeutic agents may be ideal as adjuvant therapy after curative resection if these molecules are implicated in tumor progression and metastasis. It is well known that MMPs play a key role in the development and progression of human malignancies [23–25]. MMPs mediate the destruction of the extracellular matrix, which is an important early step in tumor invasion and metastasis. In addition, it has been shown that the MMPs have angiogenic activity [26, 27] and participate in the early stages of tumorigenesis and tumor growth including metastatic tumors [28].

Our results from this retrospective study (in which the small sample size could have obscured a small benefit) indicated that disease-free survival benefit of postoperative adjuvant chemotherapy using oral fluoropyrimidines did not reach significance in tumors positive for MMP-9, contrary to the significant efficacy in tumors negative for MMP-9. Moreover, our multivariate analysis showed that MMP-9 expression in colorectal carcinoma cells was an independent factor for postoperative recurrence. The positive correlation of MMP-9 expression between the primary tumors and the recurrent liver or lung tumors indicated that MMP-9 was implicated in the development of postoperative recurrence even in secondary tumor organs. Taken together, the high MMP-9 expression in tumor was suspected to be implicated in the development of postoperative recurrence. Thus, inhibition in the MMP activities would be ideal to prevent postoperative tumor growth and secondary spreading in micrometastases. Our previous studies [29, Ogata et al.] and that of others [30] have shown that natural and synthetic MMP inhibitors inhibited the tumor vasculature, resulting in a significant decrease in hematogenous metastases in animal models, even in an adjuvant setting. Whereas recent phase III clinical studies with MMP inhibitors for advanced cancers have failed to demonstrate efficacy, the use of MMP inhibitors as postoperative adjuvant therapy has not yet been tested out in a clinical study. For future therapy of MMP inhibition, it is important to identify the relevant MMPs which must be targeted in each individual patient. It also remains to be investigated whether or not MMP's activities or expression can

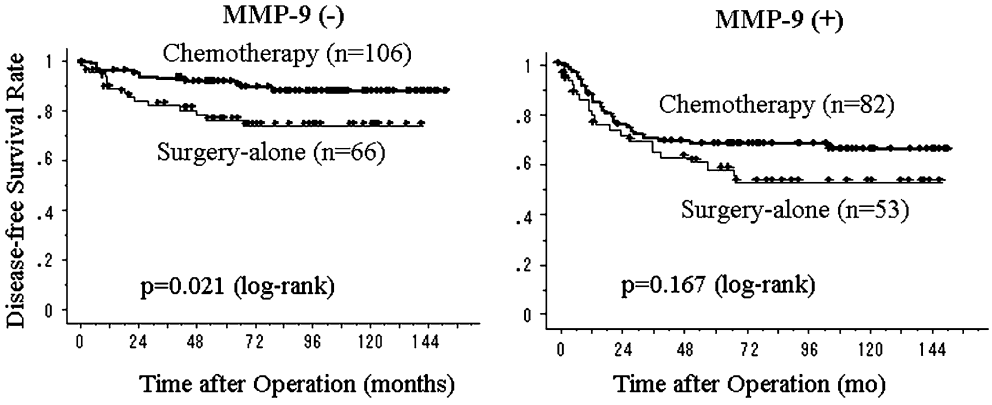

Fig. 4 The disease-free survival curves according to chemotherapy—stratified to the MMP-9 expression. There was no significant difference in the disease-free survival rate in tumors positive for MMP-9 between the chemotherapy group and the surgery-alone group ($P = 0.167$), whereas significant difference was found in those negative for MMP-9 ($P = 0.021$)

Table 4 Multivariate analysis for the factors correlated with disease-free survival rate

Variable	*P*-value	95% CI	Hazard ratio
MLN meta.	$P < 0.001$	1.058–3.698	2.571
MMP-9 (+)	$P < 0.001$	1.176–3.516	2.342
Surgery alone	$P = 0.004$	1.506–2.854	1.943
V-severe	$P = 0.036$	1.678–2.101	1.815

MLN meta metastasis to the mesenteric lymph node or lymph node belonging to the internal iliac artery. *V-severe* grade 2 or 3 venous invasion, venous invasion was defined according to the criteria of Shirouzu [11]

be a predictive marker for antitumor efficacy of MMP inhibitors in postoperative adjuvant therapy for colorectal cancer.

Recently, with regard to the efficacy of a combination of MMP inhibitors and chemotherapeutic agents, no apparent pharmacokinetic interaction between a MMP inhibitor and 5-FU has been reported in phase I studies for solid tumors including colorectal cancer [31, 32]. Moreover, it has been reported that the antimetastatic efficacy against gastrointestinal cancers was enhanced by a combination therapy of MMP inhibitor with chemotherapeutic agents such as irinotecan [33] or mitomycin C [34], without any increase in hematotoxicity. These findings support the use of oral fluoropyrimidines as a component in some combination with MMP inhibitors for adjuvant therapy in colorectal cancer patients.

In conclusion, the efficacy of postoperative adjuvant chemotherapy using oral fluoropyrimidines such as UFT and 5′-DFUR may not be great for patients with a tumor positive for MMP-9 having a greater risk of postoperative recurrence. The results support further investigation into the efficacy of MMP inhibitors in combination with oral fluoropyrimidines as postoperative adjuvant therapy for a colorectal cancer positive for MMP-9 in terms of antitumor efficacy and the patient's quality of life.

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