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Characterization of mucin 2 expression in colorectal cancer with and without chemotherapies, in vivo and in vitro study.

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ABSTRACT

Background: Colorectal cancer (CRC) is the third and second most prevalent cancer affecting males and females, respectively. 5-fluorouracil (5-FU) and irinotecan are the main chemotherapies for CRC. Down regulation of mucin 2 (MUC2) expression is associated with poor prognosis. Aim: This study examines the expression levels of MUC2 in response to treatment with 5-FU and/or irinotecan *in vivo* and *in vitro*. Method: HT29 CRC cells were treated with 5-FU and/or irinotecan, and the expression of MUC2 at mRNA and protein levels was examined by real-time polymerase chain reaction (PCR) and immunohistochemistry (IHC), respectively. CRC was induced in rats by injecting azoxymethane (AOM) prior to 5-FU treatment. Results: HT29 cells treated with 5-FU and/or irinotecan displayed a significant increase in the expression of MUC2. MUC2 mRNA expression was similar in response to monotherapy with irinotecan or 5 FU, and combinatorial treatment with irinotecan and 5-FU significantly increased MUC2 mRNA expression. Treatment of colon malignancy in rats with 5-FU resulted in increased expression of MUC2 compared with that in the positive control (rats treated with AOM only). The levels of MUC2 protein were restored and were similar to those in untreated rats. Conclusion: To our knowledge, this is the first *in vitro* study to report the effects of irinotecan treatment on MUC2 expression in CRC. MUC2 expression was increased by 5-FU and irinotecan. Therefore, further study could be undertaken to explore the potential use as a predictive marker in CRC.

1. Introduction

Colorectal cancer (CRC) refers to the abnormal growth of cells in the colon. CRC first presents as adenoma and then develops into carcinoma. This development involves a sequence of genetic mutations. Globally, it is the third and second most prevalent cancer in men and women, respectively (1). In 2017, CRC was ranked fourth in terms of cancer-related deaths (2). In Saudi Arabia, CRC is a cause of concern in younger individuals (< 50 years of age) (3, 4).

The development of CRC depends on many factors. Some examples are lifestyle—including athletic activities and dietary intake of molecules that include lipids, proteins, and vitamins—growth factors that increase the expression of insulin-like growth factor, inflammation—which is considered an important factor in Crohn's disease and ulcerative colitis—and genetic factors (5). Genes involved in CRC carcinogenesis include *APC*, *KRAS*, and *P53*, which play a role in the transition of colorectal adenoma into colorectal carcinoma (6). The *MUC* family of genes is associated with CRC (7).

CRC can be treated in several ways. Surgery is used to excise the tumor (8), radiotherapy is performed to minimize tumor size, and chemotherapy is an important approach to destroy the cancerous cells (9). The chemotherapies used in CRC treatment are anti-metabolites that include 5-fluorouracil (5-FU) (10) and topoisomerase inhibitors such as irinotecan.

5-FU is commonly used to treat CRC and acts specifically on cells in the S-phase of the cell cycle, prevents DNA synthesis (as it is analog of thymine), and inhibits enzymes that synthesize DNA (11). 5-FU is considered an efficient treatment for CRC during the early stages; however the response in late stages ranges from 10% to 15% (12). Furthermore, studies have shown that a combination of 5-FU with oxaliplatin and capecitabine is more effective than 5-FU monotherapy for treating the late stages of CRC (12, 13).

Irinotecan is another drug used in the treatment of CRC, mainly metastatic CRC, which occurs in 20% to 25% of the CRC patients (14) (14, 15). Irinotecan inhibits topoisomerase I, which normally catalyzes the formation of single-strand breaks in the DNA prior to the action of helicases. Inhibition of topoisomerase I results in the blockage of the replication process during cell cycle (16). Colon malignancies respond differently to the treatment due to the heterogeneity of the disease. Thus, it is necessary to explore both traditional and novel markers that may predict the treatment response. The markers include mucins, which are found in different organs and have multiple roles in cell proliferation, motility, and organ protection (17).

More than 20 different mucins have been reported. Some are associated with CRC. Mucins are synthesized in a multi-step process and secreted by goblet cells. The final mucin product is stored in mucous granules, from where it then can be secreted. This process protects the epithelial cells by forming a barrier between the epithelial layer and extracellular environment. Mucin also acts as a selection layer for substances that bind to epithelial cells. There are two functional types of mucins. The first types are gel-secretor mucins, which are responsible for protection of epithelial cells. These include MUC2, MUC5, and MUC6. The second types are trans-transmembrane mucins and include MUC1, MUC4, and MUC13 (17, 18).

Mucin 2 is an intestinal secretor protein encoded by the MUC2 gene. *MUC2* is considered a tumor suppressor gene and is located on chromosome 11p15.5. Mucin 2 is expressed in different amounts throughout the gastrointestinal tract, particularly in the colon (19). Mucin 2 has been known to be directly associated with CRC. Furthermore, MUC2 deficiency (Mucin 2 -/-) leads to colon inflammation and colitis, which can develop into cancer (20). Clinically, down-regulation of MUC2 expression is associated with poor prognosis (21, 22). Mutations in the *MUC2* promoter genes have

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been reported in CRC patients with worse prognosis (23). Experimental studies on MUC2 *in vitro* have revealed that down-regulation of MUC2 results in increased CRC tumorigenicity (24). Furthermore, interleukin-6 (IL-6) enhances migration and metastasis in several cancers (25). Up-regulation of MUC2 reduces the expression of IL-6 (24), which might explain the effects of MUC2 in colorectal cancer.

Although MUC2 is over-expressed in CRC cells treated with 5-FU (26), an *in vivo* study has not been performed to the best of our knowledge. The non-tumor Dark Agouti rat model of mucositis treated with irinotecan decreased the secretion of MUC2 in goblet cells of the colon (27). Similarly, tumor-bearing Dark Agouti rats treated with irinotecan showed a reduction in MUC2 expression (28). These models are not representative of the CRC model, wherein mucins might function differently.

The collective data indicate that *MUC2* plays the role of a suppressor gene in CRC. The aim of this study was to determine the expression levels of *MUC2* mRNA and protein in response to treatment with 5-FU and/or irinotecan *in vivo* and *in vitro*.

2. Materials and Methods

2.1 Ethical Approval

The animal study was approved by the Committee for the Care and Use of Laboratory Animals at Umm Al-Qura University. The study was conducted in the animal house center in the research lab of the Faculty of Medical Applied Sciences in accordance with the EU Directive 2010/63/EU for animal experiments.

2.2 Animal model

CRC was induced in 15 male Wistar rats of 10 weeks by injecting azoxymethane (AOM; 10 mg/kg/week) (Sigma-Aldrich, USA) as previously described (29). Rats were injected with AOM and 5-FU (Sigma-Aldrich, USA) in weeks 9 and 10, as previously described (30). Rats were anaesthetized using diethyl ether (Fisher Scientific, UK). The rat colons were then resected and fixed with 10% formalin overnight or preserved in RNALater (Thermo Fisher Scientific, USA) for molecular experiments. Next, fixed rat colons in 10% formalin were processed in a Leica ASP300S Fully Enclosed Tissue Processor (Leica, Germany), and embedded for immunohistochemistry (IHC). Finally, the tissues were cut into 5 µm serial sections.

2.3 Cell line

HT29 colorectal adenocarcinoma cell line (ATCC® HTB-38™) was used (American Type Culture Collection (ATCC), Manassas, VA, USA) and it was gifted from Dr Ashraf Nabil, College of Pharmacy, Umm Al-Qura University. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific, UK) containing 10% fetal calf serum and 1% penicillin/streptomycin (Invitrogen, UK). The cells were incubated at 37 °C in an atmosphere containing 95% O_2 and 5% CO_2. Cells were counted by the hemo-cytometer method. Cell suspension (50 µl) was stained with 0.4% trypan blue (Sigma-Aldrich, St. Louis, MO, USA). Cells were counted in triplicate by microscope (40×).

2.4 Cell sub-culture

Cell confluence was determined by optical microscopy. When cells were 80% to 90% confluent, they were sub-cultured by washing with phosphate buffered saline (PBS) (Thermo Fisher Scientific, UK). The washed cells were detached from the surface of the flask by treating with 2 mL trypsin EDTA (Invitrogen, UK), for 5 min. Next, 2 mL of the detached cell suspension was added to 18 mL of fresh DMEM to neutralize the trypsin EDTA. This 20 mL suspension was centrifuged at 1000 rpm for 5 min and the pellet was re-suspended in 10 mL DMEM. Then, 1 mL of the suspension was added to a flask containing 9 mL DMEM.

The suspension (cells 1.2 x 10^6) was seeded in two 6-well plates. Triplicate wells were used for the following samples: no treatment, treatment with 5-FU (stock, 50 mg/mL; working concentration, 200 µg/mL), treatment with irinotecan (stock, 20 mg/mL; working concentration, 500 µg/mL), and combination treatment with 5-FU and irinotecan. All samples were incubated for 12 h to allow the cells to reach a confluency of 80% to 90%, followed by washing, trypsin treatment, and centrifugation. Treatment protocol and chemotherapy dosage were selected from a previous study (31).

2.5 RNA extraction and cDNA synthesis

RNA was extracted using an All-Prep DNA/RNA Mini Kit (QIAGEN, UK), according to the manufacturer's instructions. RNA was measured using an ultraviolet spectrometer (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. Reverse transcription of the RNA was performed using the Maxima H Minus Reverse Transcriptase kit (Thermo Fisher Scientific,USA), according to the manufacturer's instructions. The plate was placed in the thermal cycler machine and the reaction volume was set to 20 µL. The reaction program specifications were set at 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min. The sample was stored at 4 °C until required for further analysis.

2.6 Quantitative polymerase chain reaction (qPCR) *in vitro*

Human MUC2 sequences were generated using Integrated DNA Technologies, (Leuven, Belgium). The following primers were used: glyceraldehyde 3-phosphate dehydrogenase (GAPDH, NCBI NM_017008.4) forward 5'-CATAGACACCAAAGACAAAAGC-3' and reverse 5'-GTGCTTCATCCCTGTTTTCC-3'; MUC2 (NCBI NM_002457.4) forward 5'-CCATACCCCTTAACCCCG-3' and reverse 5'-GAATTTTCACTAATGTTTCCCACC-3'. The master mix for real-time PCR contained 5 pmol primers, 10 µL of SYBR-green Master mix (Thermo Fisher Scientific, USA), and 1 µL cDNA, with DNase/RNase free water added to bring the total volume to 20 µL. The Step One device (Applied Biosystems, USA) was used to perform qPCR. The reaction process included 40 cycles of denaturation at 95 °C to separate the double helix cDNA and annealing at 60 °C to adhere the primer to its specific sequence. After 40 cycles, the automated program ran the melt curve stage.

2.7 Immunohistochemistry

IHC was performed to determine the quantity of MUC2 in CRC rat colon tissue. Endogenous peroxidase activity was blocked by adding a mixture of 600 mL methanol (MOODY, KSA) and 60 mL of hydrogen peroxide 30% H_2O_2 (Sigma-Aldrich, St. Louis, MO, USA) to prevent unwanted binding of the antibody. Tissues were incubated in the resulting 10% solution for 30 min. Normal serum was added to block the attachment of nonspecific sites by mixing 4 mL of PBS with two drops of normal serum (Vectastain ABC [avidin biotin complex] kit rabbit IgG, Burlingame, CA, USA) for a 2.5% solution. Tissues were incubated for 30 min, followed by a PBS wash. Mucin 2 rabbit polyclonal IgG (Santa-Cruz Biotechnology Inc, Santa Cruz, CA, USA) (1:150) was added and samples were incubated overnight at room temperature.

Next, 7.5 mL of PBS and 37.5 µL (0.5%) of goat anti-rabbit secondary antibody with 1% normal serum (Vectastain ABC kit rabbit IgG) was added and incubated for 30 min. ABC was prepared by washing and mixing 10 mL PBS with two drops of solution A and two drops of solution B to generate a 2% solution (Vectastain ABC kit rabbit IgG). The sections were then washed with DAP solution (9 mL of diluent and 9 drops of DAP; 5% solution) which was added to the sections for 2 min as a chromogen (Vector Laboratories, Burlingame, CA, USA). The slides were washed in distilled water, hematoxylin (Sigma-Aldrich, USA) was added, and the slides were again washed with distilled water and dehydrated. As a final step, the slides were covered with DPX mountant (Sigma-Aldrich, St. Louis, MO, USA) and examined using Leica DMi8 microscopy (Leica, Germany). The IHC staining score was determined by the H scoring system (32) to evaluate the presence and intensity of immunoreactivity. In the scoring system, the percentage of stained cells (0-100%) was multiplied by the intensity of the staining (absence=0, weak=1, medium=2, and strong=3).

2.8 Statistical analyses

Graph pad 8 software was used for statistical analysis. All data were subjected to a normality test using the Kolmogorov and Smirnov's tests. One-way ANOVA followed by post-hoc test was performed to compare the *in vitro* and *in vivo* groups. P-values <0.05 were considered significant.

3. Results

3.1 *MUC2* mRNA expression increases with adjuvant therapy *in vitro*

MUC2 mRNA expression in response to chemotherapy was determined (Figure 1). HT29 cells treated with 5-FU and/or irinotecan significantly increased expression of *MUC2* (P<0.0001) (Table 1). HT29 cells treated with 5-FU significantly increased *expression of MUC2* (P=0.0286). Similarly, compared with the expression of *MUC2* in untreated cells, cells treated with irinotecan with and without 5-FU significantly increased the expression of *MUC2* mRNA (P<0.0001). The expression of *MUC2* mRNA was almost the same in cells treated

solely with irinotecan or 5-FU, and was significantly increased when treated with both (P=0.0003).

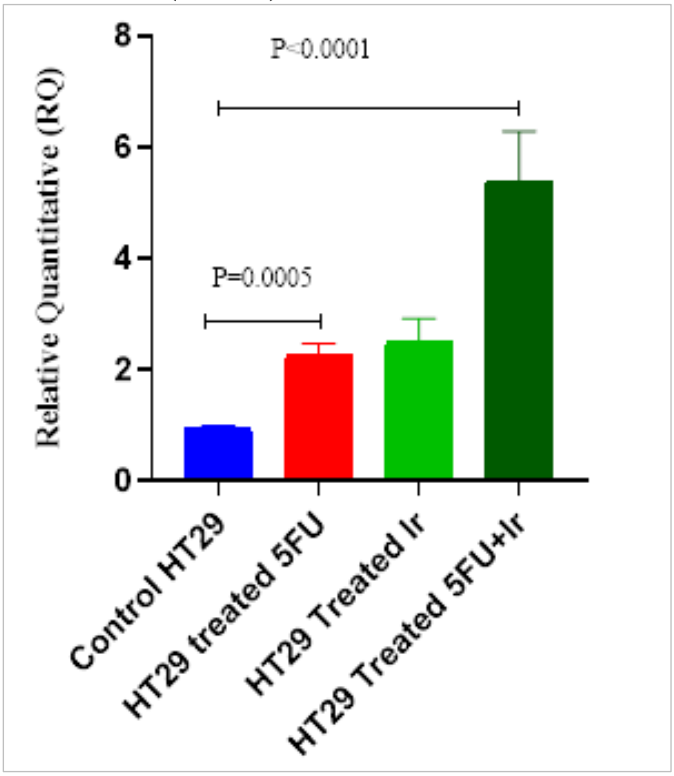

Figure 1. Relative mRNA expression of *MUC2*. The relative mRNA expression of treated HT29 CRC cells compared to a reference calibrator sample (untreated HT29 cells) by qPCR is shown. Data are presented as the ΔΔ expression of the MUC2 gene and GAPDH housekeeping gene in triplicate samples.

5-FU: 5-fluorouracil; Ir: irinotecan; CRC: colorectal cancer; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; MUC2: Mucin 2; CT: cycle threshold.

Table 1. Relative quantitative value of *MUC2* mRNA expression after chemotherapy *in vitro*. HT29 cells were treated with 5-FU and Ir. *MUC2* mRNA expression was performed by qPCR and compared by the equation ΔΔcycle threshold (CT). One-way ANOVA was used to compare between groups. Results are expressed as mean ± standard deviation.

	Control HT29	*5-FU-treated HT29*	*Ir-treated HT29*	*5-FU+ Irinotecan-treated HT29*
Mean of RQ	0.9667±0.03	2.281±0.18	2.524±0.40	5.398±0.89
Control HT29		P=0.0286 *	P=0.0133 *	P<0.0001****
HT29 treated 5-FU			P=0.8819	P=0.0003 ***
HT29 Treated Irinotecan				P=0.0005***

5-FU: 5-fluorouracil; Ir: irinotecan; MUC2: Mucin 2; RQ: relative quantitative.

3.2. MUC2 protein expression increased *in vivo* when treated with 5-FU

After studying the effect of 5-FU and irinotecan on MUC2 expression in CRC cell lines, we explored MUC2 expression *in vivo* to validate our results. AOM was injected into 10 male Wistar rats, half of which were treated with 5-FU to study the MUC2 protein expression. Normal colon specimens collected from the control group showed strong expression of mucin 2 protein (Figure 2). In the AOM group (positive control), a significant decrease (P<0.0001) in the expression of the mucin 2 protein was observed (P<0.0001) (Table 2). In rats with colon malignancy, treatment with 5-FU changed the expression pattern of mucin 2. The expression of mucin 2 increased significantly compared with the positive control (P<0.0001) (rats treated with AOM). Protein production was restored to levels similar to those in the untreated rats.

Table 2. Immunohistochemistry (IHC) score of mucin 2 protein after chemotherapy treatment *in vivo*. Mucin 2 expression was evaluated in untreated rats, positive control CRC rats (AOM treated) and CRC rats treated with 5-fluorouracil (5-FU). IHC was used to study the expression of Mucin 2. One-way ANOVA was performed to compare the groups. The data are expressed as mean ± standard deviation.

	MUC2 untreated rats	*MUC2 CRC (AOM)*	*MUC2 CRC (AOM) 5-FU*
Mean of IHC scores	84±7.9	30±15	76±16
MUC2 untreated rats		P<0.0001****	P=00.2263
MUC2 CRC (AOM)			P<0.0001****

AOM: azoxymethane; CRC: colorectal cancer; IHC: immunohistochemistry; 5-FU: 5-fluorouracil

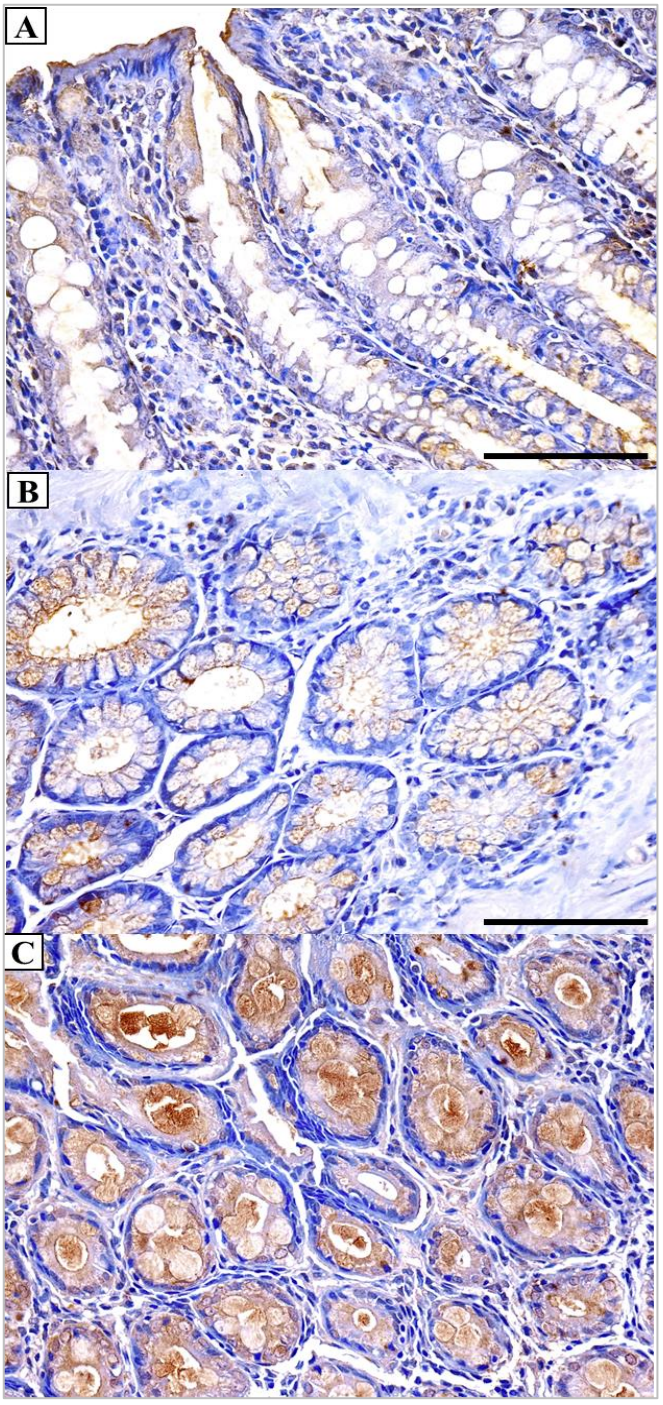

Figure 2: Immunohistochemistry localization of Mucin 2 protein in colon tissues from (A) negative control, (B) positive control, and (C) 5-FU treated group (×400 magnification; scale bar = 8 μm).

4. Discussion

CRC is one of the most prevalent cancers in Saudi Arabia. It is caused by many factors that lead to alteration in some proteins. MUC2 is one of the protective proteins affected by CRC. 5-FU and irinotecan are the main chemotherapies used to treat the late stage of CRC. In this study, the expression pattern of MUC2 was explored *in vivo* and *in vitro* by qPCR and IHC, respectively.

In vitro, expression of MUC2 increased upon chemotherapy of CRC cells. This result was consistent with the principle role of *MUC2* as a suppressor gene in CRC. Many studies have investigated the role of MUC2 in colon malignancies (21, 22, 33, 34). Mucin 2 was highly expressed in normal colon tissue. We also observed a high expression of mucin 2 in untreated rats, consistent with previous results (22). The high expression of mucin 2 in colon tissues reflects its physiological role in protecting the colon tissues (17, 18). The protective role of MUC2 in CRC could be mediated by defending colon tissues from inflammation (20), which is one of the causes of the development of CRC (35). In agreement with previous findings, mucin 2 activates the immune system signaling as overexpression of interleukin-6 (36).

In the present study, mucin 2 expression varied in the different CRC groups. Although the expression of MUC2 in mucinous CRC has been reported in several studies (37-39), the expression was reduced in non-mucinous CRC, indicating that the function of this mucin could vary between the types of CRC. In the case of *in vivo* studies, high expression of mucin 2 protein in control rats and low expression in rat CRC tissues was observed.

When the 5-FU and/or irinotecan chemotherapy regimens were performed on the samples, 5-FU treated cells displayed increased expression of MUC2 *in vivo*, and 5-FU combined with irinotecan increased the expression *in vitro*. These findings might indicate the possibility of using MUC2 as a predictable marker for analyzing treatment efficacy. Several studies have reported the association between poor outcomes and low MUC2 expression (40-45). Our results are not in agreement with those of Stringer et al. who demonstrated decreased secretion of MUC2 in non-tumor- and tumor-bearing Dark Agouti rats treated with irinotecan (27, 28). Nevertheless, these studies have not used a CRC rat model or xenograft to show the MUC2 reduction phenomena. In our study, MUC2 expression in HT-29 cells increased significantly when treated with irinotecan, which might be due to the drug enhancing a transcription factor, like those of the Cdx family (46). Furthermore, an *in vitro* study reported that MUC2 expression was decreased when 5-FU was applied (21), which agrees with the findings of our study.

This study is the first to evaluate the expression of MUC2 when CRC is treated with irinotecan. The treatment resulted in the increased expression of MUC2. Irinotecan is effective in the treatment of metastatic CRC (47). The significantly altered expression of MUC2 indicated the possibility of using MUC2 as a marker of personalized treatment. MUC2 may be useful in predicting the effects of treatment in CRC. Clinical studies will be necessary to validate these findings.

5. Conclusion

Since CRC is one of the most prevalent cancers in Saudi Arabia, a better understanding of the development of CRC, and its treatment, is thus important. Mucin 2 was highly expressed in colon tissues. The expression of the protein was reduced in malignant tissues of rats with CRC. The expression of MUC2 was restored when chemotherapy involved the treatment of 5-FU or irinotecan. To the best of our knowledge, this is the first study to report the effects of MUC2 in CRC treatment using irinotecan. The MUC2 expression patterns during CRC treatment could indicate the possibility of using MUC2 as a predictive marker of CRC treatment.

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