

Expression of inositol-requiring enzyme 1 β is downregulated in azoxymethane/dextran sulfate sodium-induced mouse colonic tumors

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Abstract. Inflammatory bowel disease (IBD) is a risk factor in colon cancer. Endoplasmic reticulum (ER) stress is associated with IBD and cancer. In the current study an azoxymethane (AOM) and dextran sulfate sodium (DSS)-induced mouse colonic tumor model was established to analyze the expression of ER stress chaperone molecules. Female C57BL/6 mice were intraperitoneally injected with 12 mg/kg AOM. On the 7th day following AOM injection, mice were treated with 1% DSS supplemented to the drinking water for 7 days, then followed by 14 days of normal drinking water. The cycle of 7 days DSS plus 14 days normal water was repeated twice and colonic tumors were evaluated for their number and size. Mice in the control group were injected with saline and received normal drinking water for the course of the experiment. mRNA levels of cytokines, inositol-requiring enzyme (IRE)1 α and 1 β , their downstream targets X-box binding protein (XBP)1 α , XBP1 β and mucin (MUC) 2 and interleukin (IL)-6, IL-8 and tumor necrosis factor (TNF)- α were detected by reverse transcription-quantitative polymerase chain reaction. IRE1 α , IRE1 β and MUC2 protein expression was evaluated by immunohistochemistry, and IRE1 α and IRE1 β levels were further assessed by western blot analysis. It was observed that tumors developed in the distal

colon of mice treated with AOM/DSS. IL-6, IL-8 and TNF- α mRNA levels were significantly increased in mice of the tumor group compared with mice of the control group. There were no significant differences in IRE1 α mRNA and protein expression between the two groups and XBP1 α mRNA levels were increased in the tumor compared with the control group. IRE1 β and MUC2 mRNA levels were significantly decreased in the tumor compared with the control group (decreased by 42 and 30%, respectively). IRE1 β and MUC2 proteins were predominately expressed in colonic epithelial cells and expression was decreased in the tumor compared with the control group. In conclusion, the downregulation of IRE1 β and MUC2 may reduce the ability of colon tissues to resist inflammation, thus promoting the occurrence and development of colonic tumors.

Introduction

Colorectal cancer (CRC) is the third most common type of cancer and the fourth-leading cause of cancer-associated mortality worldwide (1). Genetic and epigenetic genomic alterations, leading to gene amplification, the activation of oncogenes and the loss of tumor suppressor genes, are involved in the tumorigenesis of CRC (2). Risk factors associated with CRC include environmental and dietary mutagens, intestinal microorganisms and pathogens, and chronic intestinal inflammation (3). The connection between inflammation and tumorigenesis is well established. The processes of colitis-associated cancer (CAC) and non-inflammatory CRC feature common events, including the development of localized hyperplasia, polyps, adenoma and ultimately, carcinoma (4). CAC is the CRC subtype preceded by inflammatory bowel disease (IBD), Crohn's disease or ulcerative colitis (UC) (5). UC is associated with a cumulative risk of CAC of $\leq 20\%$ within 30 years of disease onset (5,6). Azoxymethane (AOM)

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is a mutagenic agent that induces multiple colonic tumors following a single injection in chronic colitis mouse models within short periods (7). This mouse model of CAC is valuable for the understanding of the mechanisms of inflammation in tumorigenesis (8).

Endoplasmic reticulum (ER) stress is associated with both the pathogenesis of intestinal inflammation and tumorigenesis (9-11). The ER is crucial for the folding of secretory and membrane proteins, lipid biosynthesis, the regulation of intracellular Ca²⁺ and redox signaling. During ER stress, the disturbance of ER homeostasis due to oxidative stress, energy deprivation, altered metabolic status or inflammatory stimuli causes ER calcium depletion and the accumulation of unfolded and misfolded proteins in the ER lumen (12). In mammalian cells, there are three types of ER stress sensors: Protein-kinase-RNA-like-ER kinase, activating transcription factor 6 and inositol-requiring enzyme (IRE) 1. These transmembrane ER proteins regulate the unfolded protein response to address ER stress (13,14). IRE1 is the most evolutionally conserved among the three ER stress response sensors (15). There are two IRE1 paralogs, α and β , present in mammals (16). IRE1 α is a ubiquitously expressed kinase and site-specific RNA endonuclease (17,18), that processes the mRNA encoding X-box binding protein (XBP) 1 to maintain ER homeostasis (19). IRE1 α excises a 26-nucleotide-intron sequence from the unspliced XBP1u mRNA to produce spliced XBP-1s mRNA, resulting in the activation of XBP1 protein expression. The XBP1 protein is a transcription factor involved in tumor growth and survival (10,20). Although IRE1 β is a paralog of IRE1 α (21), its expression is restricted to the epithelium of the gastrointestinal and respiratory tracts (16,22), while IRE1 α is expressed in a variety of tissues (23). IRE1 α and IRE1 β protect mice from dextran sulfate sodium (DSS)-induced colitis (16,24); however, these two IRE1 proteins have different functions in ER stress and varying substrate specificities (25). For example, IRE1 β has a decreased XBP1u mRNA RNase activity compared with IRE1 α (22) and IRE1 β degrades 28S rRNA more efficiently than IRE1 α (26). IRE1 β overexpression in HeLa cells results in the cleavage of 28S rRNA, leading to cell apoptosis (27). IRE1 β , but not IRE1 α , regulates the secretory protein mucin (MUC) 2 in intestinal goblet cells (26,28). MUC2 is a putatively vital molecule in the innate immune defense of the colon; a lack of the MUC2 gene results in spontaneous colitis and colon cancer in mice (29-32).

The current study induced colonic tumor formation in mice using AOM and dextran sulfate sodium (DSS). It was observed that IRE1 β and MUC2 expression was downregulated in the tumors. This indicated that the inhibition of the IRE1 β -MUC2 signaling pathway may promote the occurrence and development of colonic tumors.

Materials and methods

Mice. Female C57BL/6 (B6) mice (Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were maintained in the animal experimental center of The First Affiliated Hospital of Henan University of Science and Technology. Mice were housed with a controlled temperature of 20-22°C, 50% humidity and a 12-h light/dark cycle; they were fed with rodent chow from Beijing HFK Bioscience Co., Ltd. (Beijing, China). The Animal

Care and Use Committee of The First Affiliated Hospital of Henan University of Science and Technology (Luoyang, China) approved all the animal procedures in this study.

Colitis-mediated colon tumors were induced using a modified protocol previously described by Neufert *et al.* (7). A total of 20 mice (age, 7-8 weeks; weight, 19-23 g) were randomly allocated into the control and tumor groups (n=10/group). Mice in the tumor group received an intraperitoneal injection of 1 mg/ml AOM (12 mg/kg; molecular weight (MW), 74.08 Da; cat. no. MFCD00126912; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and control mice received saline (12 ml/kg saline). In primary experiments, the high mortality of mice was associated with direct administration following intraperitoneal injection, so on the 7th day after intraperitoneal injection of AOM, mice in the tumor group received 1.0% DSS (MW 36-50 kDa; cat. no. 160110; MP Biomedicals, LLC, Santa Ana, CA, USA) for 7 days to induce colitis. The 1% DSS solution was prepared by dissolving fine-grain DSS powder (1 g) in 100 ml drinking water. DSS solution was freshly prepared prior to administration. Water bottles containing DSS were replaced at 5 days with fresh DSS solution for the remaining 3 days. On day 8, the DSS solution was replaced with normal drinking water for 14 days; mice in the control group did not receive DSS. The cycle of 7 days DSS/14 days normal drinking water was repeated three times. Control mice were provided with normal drinking water throughout the experiment. Mice in both groups had free access to food and water. The mice were monitored once every two days for total of 69 days to record body weight, stool consistency, rectal bleeding and ulceration.

Mice were euthanized at the end of the third cycle. The entire colon was excised and measured. Colons were cut open and laid flat, lumen-side up. The number and size of colonic tumors was assessed. Colon tissues and tumor tissues were frozen at -80°C for subsequent reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analyses. RNAlater Stabilization reagent (Qiagen GmbH, Hilden, Germany) was used to prevent mRNA degradation according to the manufacturers' instructions. Further samples from the colon and tumor tissue were fixed with 10% neutral-formalin for 24 h at room temperature and embedded in paraffin 62°C for 3 h. Sections were stained by hematoxylin and eosin (H&E) and immunohistochemistry (IHC) as described below. The severity of colitis was evaluated based on the disease activity index (DAI) using a previously described method (Table I) (33).

Histopathology examination. Tissue slices (4- μ m) were prepared from distal colon tissue samples and stained with H&E for histological examination. Staining was performed at room temperature and 1% Hematoxylin (stained for 1-10 min) and 0.5% Eosin (stained for 2-5 min) were used. Pathology and level of inflammation were scored in a blinded fashion using a previously described scoring system (34) to quantify: The extent of neutrophil and lymphocyte infiltration (0-3 points); Paneth and goblet cell degranulation (0-2 points); epithelium reactivity, including crypt distortion (0-3 points); and inflammatory foci (0-3 points).

IHC was conducted with sections mounted on poly-L-lysine-coated slides using a modified biotin-peroxidase complex method as previously described (35). Briefly, tissue sections were incubated overnight at 4°C with rabbit polyclonal

Table I. Disease activity index score.

Characteristic	Assigned score				
	0	1	2	3	4
Body mass decrease (%)	0	1-5	5-10	10-15	>15
Stool appearance	Normal	-	Loose	-	Diarrhea
Rectal bleeding	Normal	-	Hemoccult positive	-	Gross bleeding

The disease activity index=(body mass decrease + stool character + rectal bleeding)/3.

antibodies against IRE1 α (dilution, 1:150; cat. no. sc-20790; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), IRE1 β (dilution, 1:100; cat. no. GTX87426; GeneTex, Inc., Irvine, CA, USA) and MUC2 (dilution, 1:100; cat. no. ab76774; Abcam, Cambridge, UK). Antigen-antibody complexes were detected with a biotinylated goat anti-rabbit antibody (dilution, 1:300; cat. no. SA1020; Boster Biological Technology Co., Ltd., Wuhan, China) conjugated with streptavidin-horseradish peroxidase (HRP) for 30 min at 37°C. At room temperature and visualized by reacting with the 3,3'-diaminobenzidine reagent kit (Solarbio Science & Technology Co., Ltd., Beijing, China; cat. no. DA1010) at room temperature for 5 min. Sections were counterstained with 1% hematoxylin for 1-10 min at room temperature. Negative control sections were obtained by omitting the primary antibody or by using an unspecific antibody. Images at a magnification of x400 were captured using a Nikon Ds-Fi2 500w light microscope, (Nikon Corporation, Tokyo, Japan). IHC staining was used to semi-quantitatively determine protein levels as previously described (35): A total of 10 fields were randomly selected on each slide and 100 cells per field were counted and scored for IRE1 α , IRE1 β and MUC2 staining. The semi-quantitative scores were obtained from the staining intensity (none, 0; weak, 1; moderate, 2; strong, 3) multiplied by the percentage of positively stained cells ($\leq 5\%$, 0; 6-25%, 1; 26-50%, 2; 51-75%, 3; and >75%, 4).

RT-qPCR analysis. Total RNA from the colon tissue samples was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. cDNA was synthesized using PrimeScript-RT Master mix (Takara Bio, Inc., Otsu, Japan) with the following temperature protocol: 37°C for 15 min, 85°C for 5 sec and 4°C for 10 min. qPCR was performed as described previously, using undiluted cDNA templates (35). Primer sequences (Table II) were designed with Primer 3.0 (36) and synthesized by Sangon Biotech Co., Ltd. (Zhengzhou, China). qPCR was performed using a CFX96 Real-Time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and SYBR green (Takara Bio, Inc.) was used to detect PCR products. The reaction followed an initial step at 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec, 58°C for 30 sec and 72°C for 30 sec. For annealing temperatures >60°C, a two-step method was used (37). Each sample was assayed in triplicate. Amplification efficiency was >97%. A melting curve analysis was performed for the PCR products of each target gene and β -actin to evaluate primer specificity. The relative abundance of target gene

mRNA level was evaluated using $2^{-\Delta\Delta Cq}$ method, normalized to β -actin (38). mRNA expression levels were presented as relative fold by comparing the quantity of mRNA between different groups. When $Cq > 35$, the gene was considered to not be expressed.

Western blotting. Protein lysates were prepared from the extracted tissues in radioimmunoprecipitation assay lysis buffer (cat. no. R0020; Solarbio Science & Technology Co., Ltd.) on ice by homogenization with a grinder. The supernatant was obtained by centrifugation (15 min; 10,800 x g; 4°C. The bicinchoninic acid (cat. no. PC0020; Solarbio Science & Technology Co., Ltd.) method was used to determine protein concentration. Protein (30 μ g) from each sample was denatured, resolved on 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Rabbit polyclonal antibodies against IRE1 α (dilution, 1:1,000; cat. no. 14C10; Cell Signaling Technology, Inc., Danvers, MA, USA) and IRE1 β (dilution, 1:300; cat. no. ab135795; Abcam) and mouse monoclonal antibodies against GAPDH (dilution, 1:1,000; cat. no. CW0100A; CWBIO Technology Co. Ltd, Beijing, China) were incubated with the membranes at 4°C overnight, followed by HRP-conjugated anti-rabbit (dilution, 1:1,000; cat. no. BA1054) or anti-mouse IgG (dilution, 1:3,000; cat. no. BA1050; both Boster Biological Technology Co. Ltd.) for 1 h at room temperature, and enhanced chemiluminescence reagent (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturers' protocols. GAPDH was used to normalize protein expression. A ChemiDoc XRS (Bio-Rad Laboratories, Inc.) was used to capture images, which were quantified using ImageJ software v1.48 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All data are presented as the mean \pm standard deviation. Experiments were repeated at least three times. All statistical analyses were performed using SPSS 20.0 (IBM Corp., Armonk, NY, USA). Student's t-test or Mann-Whitney U test were used to determined significant differences between groups. Wilcoxon signed-rank test was used for non-parametric data. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Treatment with AOM/DSS induces tumors in mouse colons. According to Thaker *et al* (39) and preliminary experiments, the most consistent results were observed using female mice;

Table II. Reverse transcription-quantitative polymerase chain reaction primers.

Gene	Primer (5'-3')	
	Forward	Reverse
IL-6	AACGATGATGCACTTGCAGA	TGGTACTCCAGAAGACCAGAGG
TNF- α	CCACCACGCTCTTCTGTCTACT	TGCTACGACGTGGGCTACA
IL-8	CTAGGCATCTTCGTCCGTCC	TTGGGCCAACAGTAGCCTTC
XBP1u	GGTCTGCTGAGTCCGCAGCACTC	AGGCTTGGTGTATACATGG
XBP1s	GGTCTGCTGAGTCCGCAGCAGG	AGGCTTGGTGTATACATGG
IRE1 α	GCATCACCAAGTGGAAAGTATC	ACCATTGAGGGAGAGGCATAG
IRE1 β	CACAACCTATCGCCGCTACT	CATCCTGGTGCCATGTGTAA
MUC2	CTGACCAAGAGCGAACACAA	CATGACTGGAAGCAACTGGA
β -actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

IL, interleukin; TNF, tumor necrosis factor; IRE, inositol-requiring enzyme; XBP, X-box binding protein; MUC, mucin.

hence, only female mice were utilized in the current study. No signs of inflammation or colonic tumors were observed in the control group. Mice in the tumor group exhibited no sign of illness following AOM treatment and reduced appetite and drank less starting on day 6 of the 1% DSS treatment. By day 7, all mice of the tumor group presented with bloody stools and declining body weight; symptoms lasted until day 4 after 1% DSS treatment. The weight loss of the mice in the tumor group was the lowest with -5.5% during the first cycle. In the following two cycles, mice exhibited similar symptoms, but less severe. Body weights of mice in the tumor group increased, although slower compared with the control mice; particularly during DSS treatment. At the endpoint, there was a significant difference in body weight between the tumor and the control group ($P < 0.05$; Fig. 1) and DAI scores in the tumor group were significantly higher compared with the control group ($P < 0.05$; Table III). No mortality was observed in either group. The colon length in the tumor group was significantly decreased compared with the control group ($P < 0.05$; Table III). A total of 36 (average number of tumors per mouse, 3.6; range, 2-5) colonic tumors were observed in mice of the tumor group. The mean tumor diameter was 3.1 mm (range, 1-4 mm). Histological examination revealed that the mucosa of the colons from the tumor group were disordered and the crypt structure was destroyed compared with the control group: Colonic epithelial cells were atypical, with decreased differentiation, increased cell size and large nuclei; the nucleus-to-cytoplasm ratio of the cells was increased, the number of goblet cells was decreased and the number of inflammatory cells was increased (Fig. 2A and B).

mRNA expression differs between the control and tumor groups. mRNA expression of IL-6, IL-8 and TNF- α was determined in colonic tissues from the two groups. IL-6, IL-8 and TNF- α mRNA expression was significantly increased in the tumor compared with the control group (17.6-, 32.3- and 5.9-fold, respectively; $P < 0.05$; Fig. 3).

IRE1 α and XBP1u mRNA expression levels were not significantly different between the tumor and the control group ($P > 0.05$; Fig. 4). XBP1s mRNA levels were significantly increased in the tumor compared with the control group

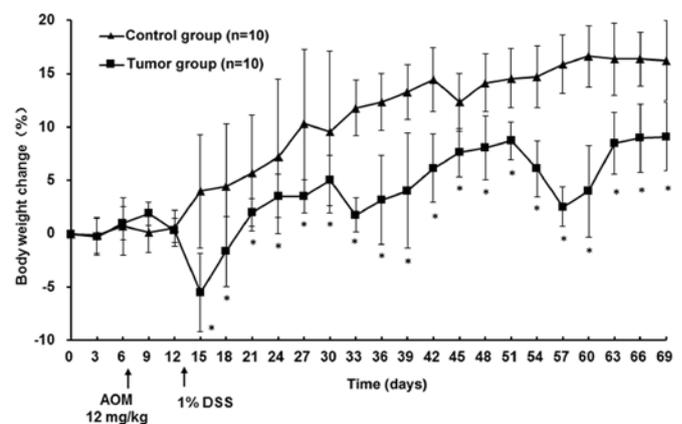


Figure 1. Changes in body weight of the mice over the course of the experiment. At day 1, mice in the tumor group received an intraperitoneal injection of azoxymethane. On 7th day mice in the tumor group received drinking water containing 1% dextran sulfate sodium for 7 days, followed by normal drinking water for 14 days. This 7/14 day cycle was repeated three times. Mice in the control group were injected with saline and received normal drinking water for the duration of the experiment. * $P < 0.05$ vs. control group.

(~2-fold; $P < 0.05$; Fig. 4) and IRE1 β and MUC2 mRNA levels in the tumor group were significantly decreased compared with the control group (42 and 30%, respectively; $P < 0.05$; Fig. 4).

IRE1 β and MUC2 protein expression is downregulated in tumor tissues. As determined by IHC staining, there was no significant difference in IRE1 α protein expression between the tumor and control group ($P > 0.05$; Table IV). IRE1 α was primarily expressed in the cytoplasm of colonic submucosa cells. A low level of IRE1 α expression was observed in the cytoplasm of colonic epithelial cells, particularly in brush border cells (Fig. 2C and D). IRE1 β protein expression in the tumor group was significantly decreased compared with the control group ($P < 0.05$; Table IV). IRE1 β protein was predominantly expressed in the cytoplasm of colonic mucosa epithelial cells (Fig. 2E and F). Similar to IRE1 β , the expression of MUC2 protein in the tumor group was significantly lower than in the control group ($P < 0.05$; Table IV). MUC2 protein

Table III. Disease activity index scores and colon length.

Group	N	DAI	Colon length (cm)
Control	10	0	8.7±0.6
Tumor	10	3.3 ^a	6.4±0.6 ^a

^aP<0.05 vs. control group. DAI, disease activity index.

Table IV. IRE1 α , IRE1 β and MUC2 protein expression in colonic tissues determined by immunohistochemistry analysis.

Group	N	IRE1 α	IRE1 β	MUC2
Control	10	1.50±0.23	5.26±0.38	4.77±0.47
Tumor	10	1.63±0.18	2.53±0.23 ^a	1.83±0.41 ^a

^aP<0.05 vs. control group. IRE, inositol-requiring enzyme; MUC, mucin.

was primarily expressed in the cytoplasm of colonic mucosa epithelial cells, particularly in goblet cells in the normal mucosa (Fig. 2G and H).

The results of western blot analysis revealed no significant difference in the expression of IRE1 α protein between the groups (Fig. 5A). IRE1 β protein expression was significantly decreased in the tumor compared with the control group (P<0.05; Fig. 5B).

Discussion

IRE1 α serves a protective role against ER stress and colitis (24,40). The IRE1-XBP1 signaling pathway serves a pivotal role in cell survival under conditions of ER stress and is associated with tumorigenesis of various cancer types (41-44). In the current study, mRNA expression of IRE1 α , XBP1u and XBP1s and IRE1 α protein expression were compared in tumor and normal tissues. XBP1 mRNA, a signaling molecule in the IRE1 α -XBP1 signaling pathway, is processed by IRE1 α (19). XBP1s mRNA expression was significantly increased in tumor compared with control tissues. This suggested that the activity of the IRE1 α -XBP1 pathway was increased, indicating that the ER stress response may be associated with tumorigenesis. The present study further observed that IRE1 α mRNA levels were not increased in tumor tissues compared with the control, while XBP1s mRNA levels were. Under ER stress, IRE1 α is activated through trans-autophosphorylation and dimerization, or oligomerization (45). It was speculated that activated IRE1 α levels may be increased, while gene expression levels remained unchanged. Thus, the determination of phosphorylated-IRE1 α levels requires future assessment.

IRE1 α is a transmembrane RNase that initiates the splicing of XBP1u to XBP1s. The latter encodes a transcription activator; its activity induces the expression of ER chaperones (46). IRE1 α promotes cell survival-associated signal transduction and the transmission of apoptotic signals (47). It has been demonstrated that the IRE1-XBP1 signaling pathway is associated to the

pathogenesis of IBD (48,49) and human cancer (50), leading to the consideration of the IRE1-XBP1 signaling pathway as a potential target for cancer therapy (51). A recent study suggested that IRE1 α promotes cell survival by splicing XBP1 mRNA and promoting regulated IRE1-dependent decay (RIDD). During the RIDD process, IRE1 promotes the degradation of mRNAs that predominately encode ER-targeting proteins (52), which may be the mechanism for the involvement of the IRE1 α -XBP1 signaling pathway in the pathogenesis of inflammation-mediated CRC. It was previously reported that IRE1 α and XBP1 protein expression levels were unchanged in human CRC tissue compared with adjacent normal tissue (44). Therefore, the role of the IRE1-XBP1 signaling pathway in the pathogenesis of colonic tumors may be discrepant between humans and mice.

IRE1 β , a homolog of IRE1 α , protects from colitis and IRE1 β ^{-/-} mice are more sensitive to experimentally induced colitis (16). In contrast to IRE1 α , IRE1 β affects different substrates, regulating the mRNA that encodes ER proteins to maintain ER homeostasis in highly differentiated secretory cells (28). Divergent effects of IRE1 α and IRE1 β on the cell fate may be implicated by their varying roles in tumorigenesis (25,46). Therefore, the current study analyzed IRE1 β expression in tumor tissues. The results suggested that IRE1 β mRNA and protein levels were decreased in the tumor compared with the control tissues. Although Tsuru *et al* (28) reported that IRE1 β is specifically expressed in the ER of goblet cells, present IHC results revealed that IRE1 β positive staining was observed in goblet and absorptive cells, indicating that IRE1 β may affect multiple cells types. IRE1 β acts to protect the intestine from inflammation (52). IRE1 β expression in the epithelium of the gastrointestinal tract is consistent with promoting resistance to DSS-induced colitis at the epithelial cell level (16). When DSS is ingested, the gastrointestinal tract epithelial cells are the first to be exposed to the treatment (16). Toxicity likely has a primary role in the development of inflammation among colonic epithelia. DSS acts on the epithelial cell barrier, leading to reduced IRE1 β expression in epithelial cells and weakening the protection against colonic inflammation (16). As IRE1 β is a protective factor in colitis that can induce apoptosis (23) it is expected that a decrease in IRE1 β expression is associated with tumorigenesis, as apoptosis is putatively associated with tumor occurrence (53). IRE1 β has been reported to regulate the expression of microsomal triacylglycerol transfer protein, a regulator of lipid absorption in the colonic epithelium (54); however, it is unclear whether this function of IRE1 β affects tumorigenesis. In an *in vitro* study, Dai *et al* (55) reported that IRE1 β mRNA expression increases in undifferentiated and decreases in differentiated Caco-2 cells. IRE1 β has been revealed to be involved in cell apoptosis (27). In a previous study, it was reported that IRE1 β and MUC2 expression are downregulated in human colonic tissues (44). These results indicated that IRE1 β serves an important role in the pathogenesis of colitis and tumors.

MUC2 is the major component of the intestinal mucus gel barrier, which serves an important role in the protection of intestinal function (31). Furthermore, MUC2 is putative to protecting the intestine against colitis and colorectal cancer (30). It has been demonstrated that MUC2-deficient mice spontaneously develop colon cancer (32). Consistent with this, the results of the current study revealed that MUC2

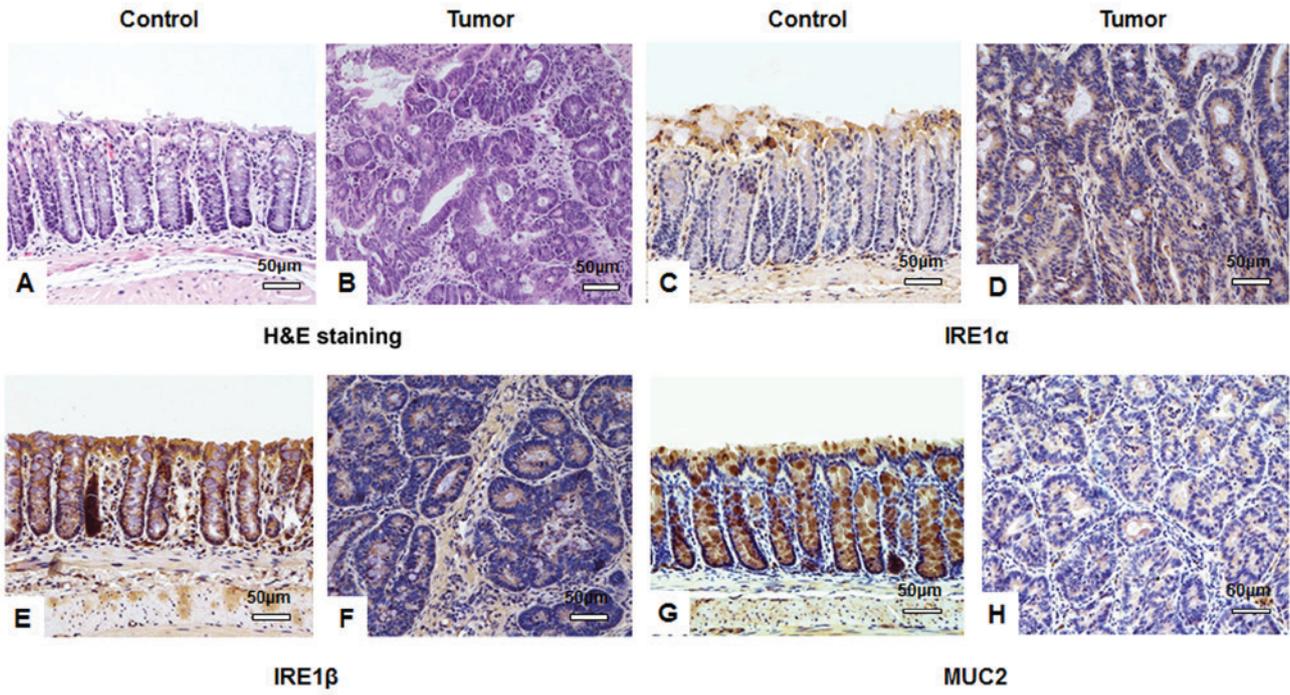


Figure 2. IHC staining suggests that AOM/DSS treatment affects IRE1 α , IRE1 β and MUC2 expression in tissues. Following a one-off treatment with AOM and three cycles of DSS, tumor tissues from mice in the tumor group (n=10) and colon tissues from mice in the untreated control group (n=10) assessed by IHC. Hematoxylin and eosin staining of (A) control and (B) tumor tissues identifying typical tumor characteristics, including disordered mucosa, destroyed crypt structure, less differentiated cells, a larger cell and nuclei size, and an increased nucleus-to-cytoplasm ratio. IRE1 α protein was stained using IHC in (C) control and (D) tumor tissues and expression in the cytoplasm of colon submucosa cells was observed. IHC visualized IRE1 β protein in (E) control and (F) tumor tissues and identified cytoplasmic expression in colonic mucosa epithelial cells. MUC2 protein expression was visualized using IHC in goblet cells of (G) control and (H) tumor tissues. All images were obtained using a Nikon Ds-Fi2 500w [Ds-Fi2; light microscope (magnification, x200)]; scale bar, 50 μ m. IHC, immunohistochemistry; AOM, azoxymethane; DSS, dextran sulfate sodium; IRE, inositol-requiring enzyme; MUC, mucin.

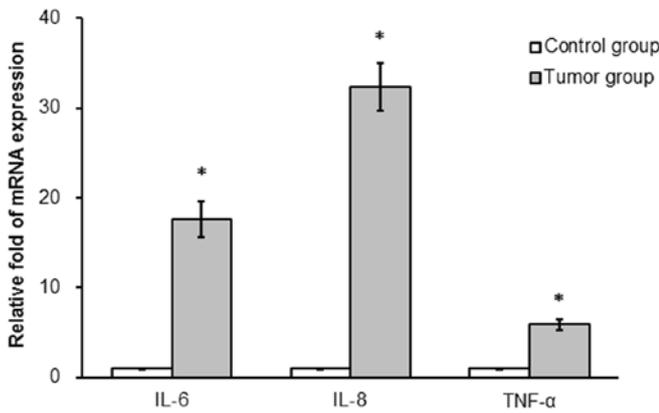


Figure 3. AOM/DSS treatment increases mRNA levels of inflammatory cytokines in mice. Following a one-off treatment with AOM and three cycles of DSS, IL-6, IL-8 and TNF- α mRNA expression levels were determined in mice of the tumor and the untreated control group (n=10/group) using reverse transcription-quantitative polymerase chain reaction assays. *P<0.05 vs. control group. AOM, azoxymethane; DSS, dextran sulfate sodium; IL, interleukin; TNF, tumor necrosis factor.

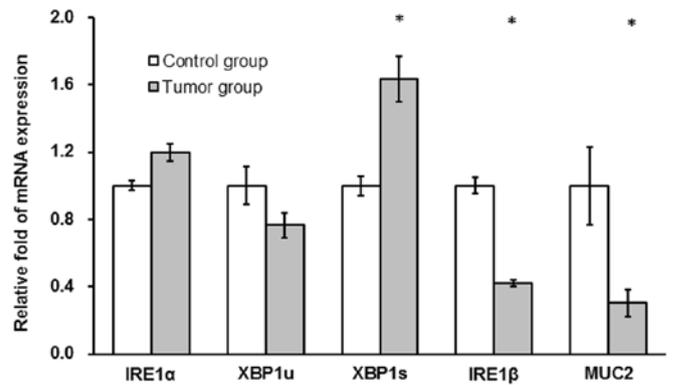


Figure 4. AOM/DSS treatment affects mRNA levels of XBP1s, IRE1 β and MUC2 in colon tissues of mice. Following a one-off treatment with AOM and three cycles of DSS, mRNA expression of IRE1 α , XBP1u, XBP1s, IRE1 β and MUC2 were determined in tissues from mice in the untreated control and tumor groups (n=10/group) at the end point of the experiment using reverse transcription-quantitative polymerase chain reaction assays. *P<0.05 vs. control group. AOM, azoxymethane; DSS, dextran sulfate sodium; IRE, inositol-requiring enzyme; XBP, X-box binding protein; MUC, mucin.

mRNA and protein levels were significantly decreased in tumor compared with normal tissue. It has been reported that IRE1 β is involved in the ER homeostasis of colon goblet cells and the synthesis and secretion of MUC2 (28). IRE1 β is believed to control the levels of translatable cytosolic MUC2 mRNA to maintain MUC production (28). An *in vitro* study has revealed that IRE1 β regulates secretory proteins in cells via degrading

mRNA in the ER (26). In addition, IRE1 β is essential for airway epithelial MUC production (22). These data suggest that IRE1 β may regulate MUC2 expression and the downregulation of IRE1 β and MUC2 may reduce the protection of the colon to promote occurrence and development of tumors.

The current findings suggested that the ER stress-associated IRE1 α -XBP1 signaling pathway was activated during

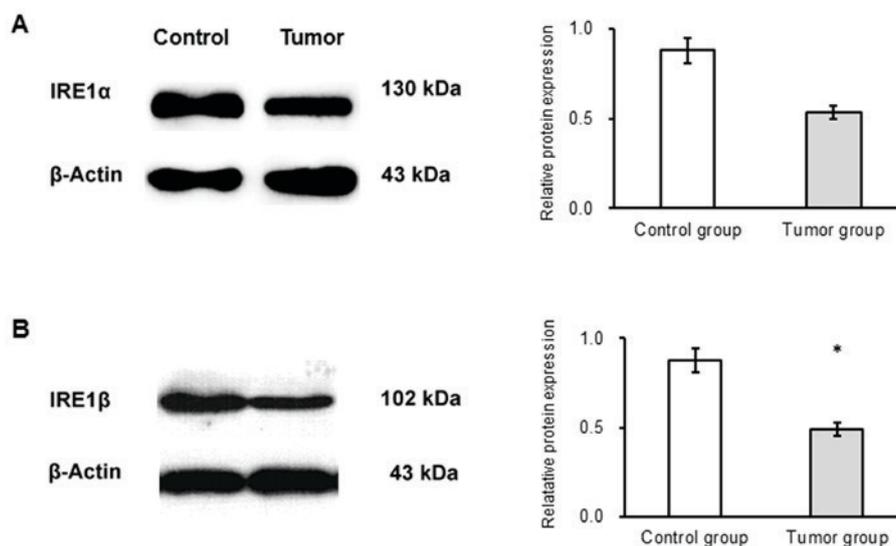


Figure 5. IRE1 α and IRE1 β protein expression is decreased in mice receiving AOM/DSS treatment. Following a one-off treatment with AOM and three cycles of DSS, tumor tissues from mice in the tumor group (n=10) and colon tissues from mice in the untreated control group (n=10) were collected. (A) IRE1 α and (B) IRE1 β protein expression was assessed by western blotting. The reduction of IRE1 α expression was not significant, but the expression of IRE1 β decreased significantly. *P<0.05 vs. control group. AOM, azoxymethane; DSS, dextran sulfate sodium; IRE, inositol-requiring enzyme.

the occurrence and development of CRC, and potentially contributed to these processes. The decreased expression of two protective molecules, IRE1 β and MUC2, promoted the occurrence and development of inflammation and ultimately tumors in the colonic epithelium. The mechanism of these processes requires further analysis to identify potential therapeutic targets for the treatment or prevention of colitis-associated CRC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FD performed the majority of experiments, analyzed the data and drafted the manuscript. SD, QX, PC and MC provided technical support, purchased reagents and analyzed the data. ZR and YF analyzed the data and provided their guidance for the study and manuscript writing. QG provided funding, designed the current study, analyzed the data and wrote the manuscript.

Ethics approval and consent to participate

The Animal Care and Use Committee of The First Affiliated Hospital of Henan University of Science and Technology approved all the animal procedures in this study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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