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Expression of inositol-requiring enzyme 1β is downregulated in colorectal cancer

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Abstract. The endoplasmic reticulum stress inositol-requiring enzyme (IRE) 1α/X-box binding protein (XBP) 1 signaling pathway is involved in the tumorigenesis of breast and prostate cancer. Mucin 2 (MUC2) protects colon tissues from the formation of tumors. In human colorectal cancer (CRC) the role of IRE1α, and its analogue, IRE1β, has yet to be elucidated. In the present study, the expression levels of IRE1α, IRE1β, un-spliced XBP1u, spliced XBP1s and MUC2 in surgically resected cancerous and adjacent non-cancerous tissues from patients with CRC were investigated. The IRE1α, IRE1β, XBP1u, XBP1s and MUC2 mRNA expression levels were determined using reverse transcription-quantitative polymerase chain reaction, and the protein expression levels were detected using immunohistochemistry and western blotting. The association between the expression levels of IRE1α, IRE1β and MUC2 and the clinicopathological features of patients with CRC was subsequently analyzed. The mRNA expression levels of IRE1β and MUC2 were decreased by ~2.1 and ~4.5-fold in CRC tissues, respectively, as compared with the adjacent normal tissues. The protein expression levels of IRE1β and MUC2 were decreased by ~8.0 and ~2.0-fold in the CRC tissues, respectively. IRE1β mRNA expression levels were positively correlated with MUC2 mRNA expression levels. IRE1β expression levels were revealed to be significantly associated with lymph node metastasis, tumor stage and histological differentiation. However, IRE1α, XBP1u and XBP1s mRNA and IRE1α protein expression levels were not observed to significantly differ between cancerous tissues and the adjacent normal tissues. The results indicated that the expression of IRE1β, but not IRE1α, may protect colon tissue from developing CRC by inducing MUC2 expression. Therefore, decreased IRE1β expression levels may be associated with the development of CRC through the inhibition of MUC2 expression.

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Abbreviations: ATF6, activating transcription factor 6; CRC, colorectal cancer; ER, endoplasmic reticulum; IRE1, inositol-requiring enzyme 1; MUC, mucin; PERK, protein-kinase-RNA-like-ER kinase; RIDD, regulated IRE1-dependent decay; UPR, unfolded protein response; XBP1, X-box binding protein 1

Key words: colorectal cancer, endoplasmic reticulum stress, inositol-requiring enzyme 1, mucin 2, X-box binding protein 1

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second leading cause of cancer-associated mortality in the United States (1). In China, CRC is the fifth leading cause of cancer-associated mortality, following lung, liver, gastric and esophageal cancer (2). The incidence of CRC in China has increased in recent decades and is predicted to continue rising due to changes in lifestyle and diet (3,4). Although novel treatments have been developed, the five-year survival rate of patients with CRC and distant metastases remains poor at ~13% (5-7). CRC develops from the accumulation of genetic and epigenetic alterations, leading to gene amplifications, the activation of certain oncogenes or the loss of tumor suppressor genes (6).

Previous studies have demonstrated that the activation of endoplasmic reticulum (ER) stress in cancer cells may facilitate their survival and tumor growth; however, certain studies have revealed that ER stress may inhibit cancer progression (8,9). During ER stress, various pathological changes occur to induce ER calcium depletion and the accumulation of misfolded proteins in the ER lumen (10). Mammalian cells have three classes of ER stress sensors, including protein-kinase-RNA-like-ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme-1α (IRE1α) (10-12). These sensors are resident ER transmembrane proteins, which regulate the unfolded protein response (UPR) to manage ER stress (11). The UPR includes

the alteration of protein folding, assembly and degradation programs in order to reestablish homeostasis and normal ER functioning (11,13).

IRE1α has a dual enzyme activity, as it is a kinase and a site-specific RNA endonuclease (8,14). IRE1α is frequently mutated in various types of human cancer (15). One manner in which IRE1α maintains ER homeostasis is by processing the mRNA encoding X-box binding protein 1 (XBP1) (16). IRE1α activates XBP1 protein expression by excising a 26-nucleotide-intron sequence from the un-spliced XBP1 mRNA (XBP1u) and creating the spliced XBP1 mRNA (XBP1s). The subsequent frame shift mutation eliminates a stop codon for protein translation (17,18). XBP1 is a transcription factor that is involved in tumor growth and survival (9,17). XBP1 expression levels are increased in numerous types of cancer, including breast cancer (19), hepatocellular carcinoma (20), pancreatic cancer (17), and CRC, as determined in a study of five patients (21); however, XBP1 expression levels may be decreased in prostate cancer (22). IRE1α may also induce UPR through the post-transcriptional modifications of specific ER membrane proteins via regulated IRE1-dependent decay (RIDD) (8). RIDD is the process by which IRE1α promotes the degradation of mRNAs primarily encoding ER-targeted proteins, in order to reduce the influx of proteins during ER stress (23,24). In the present study, IRE1α and XBP1 mRNA expression levels in CRC tissues were analyzed to determine whether they are increased, compared with the paired control samples.

IRE1β is an analog of IRE1α (25). Whereas IRE1α is expressed ubiquitously, the expression of IRE1β is restricted to the epithelium of the gastrointestinal and respiratory tracts (26,27). Although previous studies have demonstrated that IRE1α and IRE1β are each able to sense ER stress and protect mice from dextran sulfate sodium-induced colitis (26,28), they have differing functions. The two IRE1 proteins have various substrate specificities; the RNase activity of IRE1α with regard to XBP1u mRNA is markedly high, compared with IRE1β (27). IRE1α directs cell survival through the induction of XBP1 mRNA cleavage and the promotion of RIDD (28). IRE1α signaling terminates in the event of cell apoptosis induced by irremediable ER stress (29-31). IRE1β is more efficient than IRE1α at degrading 28 s rRNA (16). The cleavage of 28s rRNA may induce apoptosis, as previously demonstrated in IRE1β-overexpressing HeLa cells (32). The RNase activity of IRE1β appears to have broad substrate specificity; it regulates the stability of the mRNA that encodes certain ER proteins and maintains ER homeostasis in highly differentiated secretory cells (16). Thus, IRE1β, but not IRE1α, degrades the mRNA encoding specific secretory proteins, including mucin 2 (MUC2) in the intestinal goblet cells (16,33). MUC2 is a macromolecular glycoprotein secreted by goblet cells (34). MUC2 is crucial to host immune system and protects colon tissues from developing colitis or CRC; MUC2-deficient mice have been observed to develop spontaneous colitis and colon cancer (35-38). The differences between the substrates of IRE1α and IRE1β suggest their divergent functions in ER stress, and may also reflect their various roles in the tumorigenesis of CRC (29). Therefore, IRE1β and MUC2 gene expression profiles in CRC tissue samples were analyzed in the current study.

In the present study, the expression levels of the signaling pathways IRE1α-XBP1, IRE1β and MUC2 in colon cancer tissues were investigated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), western blotting and immunohistochemistry, and their associations with the clinical features of CRC patients were explored. This study may identify potential important targets for cancer therapies.

Materials and methods

Patients and tissue samples. The CRC tissue samples were obtained from surgically resected tumor tissues from patients with colorectal adenocarcinoma at the First Affiliated Hospital of Henan University of Science and Technology, between September 1st, 2013 and February 31st, 2014. The clinicopathological features of the patients recruited for the current study are listed in Table I. Tumor tissues and adjacent non-cancerous tissues (serving as controls) were analyzed. The control non-cancerous tissues were taken from an area ~5 cm from the lesion. Patients with colorectal adenocarcinoma were selected, while those patients with mucinous, signet-ring cell carcinoma, squamous carcinoma, adenosquamous carcinoma or undifferentiated carcinoma forms of CRC were excluded. Mucinous CRC was excluded due to its high level of MUC2 expression compared with that in normal colon tissues (36,39,40).

Two staff pathologists confirmed the diagnosis of CRC. A section of each tissue sample was fixed in 4% paraformaldehyde and embedded in paraffin wax for hematoxylin and eosin staining and immunohistochemistry (IHC). The remaining section of the tissue sample was stored at -80˚C for RNA extraction and western blot analysis. RNA*later* (Qiagen GmbH, Hilden, Germany; cat. no. 76106) was added immediately following the tissue sample collection in order to prevent RNA degradation. The tumor stages were classified according to the 7th edition of the tumor-node-metastasis (TNM) classification criteria of the American Joint Committee on Cancer (41). Informed consent was obtained from all patients and the Clinical Research Ethics Committee of The First Affiliated Hospital of Henan University of Science and Technology approved the current study.

RT-qPCR. Total RNA was extracted using TRIzol® Reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. A total of 2 μg total RNA was used for cDNA synthesis using PrimeScript™ RT Master Mix (Takara Bio, Inc., Otsu, Japan) in a 40 μl reaction mixture (8 μl 5X RT Master Mix; total RNA; diethylpyrocarbonate), as follows: 37˚C for 15 min, 85˚C for 5 sec and 4˚C for 10 min. The primer sequences for IRE1α, XBP1u, XBP1s, IRE1β, MUC2 and β-actin were designed using Primer3.0 software (42) and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China; Table II). RT-qPCR was conducted using a CFX96™ Real-Time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The reaction mixture (25 μl total volume per well) included 2 μl cDNA, 12.5 μl 2xSYBR Premix Ex *Taq* II (Takara Bio, Inc.), 8.5 μl H_2O and 2 μl 0.4 μM primers. A two-step method was used due to the 60˚C annealing temperature. The reaction consisted of the following: 95˚C for 30 sec, 40 cycles of 95˚C for 5 sec and

60˚C for 30 sec. Each tissue sample was assayed in triplicate. The efficiency of the PCR amplification process was 97-105%. A melting curve analysis was performed for the PCR products of the target genes in order to evaluate primer specificity. Relative quantification of the target gene mRNA expression was conducted using quantification cycle (Cq) with the formula $\log_{10}2^{-\Delta\Delta Cq}$ (43) and normalized to β-actin. The difference in mRNA expression was presented as the relative fold between the groups. A Cq value of >35 was considered to indicate that a specific gene was not expressed.

Immunohistological staining for IRE1α, IRE1β and MUC2. Sections (4 μm) of paraffin-embedded tissue samples were mounted on poly-L-lysine-coated slides. IHC was performed using an indirect peroxidase-labelled antibody method as previously described (44). Briefly, the tissue sections were dewaxed in xylene, rehydrated with graded alcohol and antigen retrieval was conducted by microwave-boiling the slides for 10 min in 0.01 mol/l sodium citrate buffer, pH 6.0. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide for 10 min. Following washing in distilled water, non-specific binding was blocked by 5% bovine serum albumin (Sigma-Aldrich: Merck Millipore, Darmstadt, Germany) for 20 min at 37˚C. The tissue sections were then incubated overnight at 4˚C with rabbit polyclonal antibodies against IRE1α (Abcam, Cambridge, UK; dilution, 1:200; cat. no. ab37073) and IRE1β (Abcam; dilution 1:50; cat. no. ab135795), and an anti-MUC2 mouse monoclonal antibody (Abcam; dilution, 1:500; cat. no. ab11197). The antigen-antibody complex was then detected with a biotinylated goat anti-rabbit antibody (Boster Biological Technology Co., Ltd., Wuhan, China; cat. no. BA1101) and an anti-mouse antibody (Boster Biological Technology Co., Ltd.; cat. no. SA1020), subsequently conjugated with streptoavidin-horseradish peroxidase (HRP) (Boster Biological Technology Co., Ltd.; cat. no. SA1022) and visualized by reacting with nickel-enhanced 3,3-diaminobenzidine tetrahydrochloride (Solarbio Science and Technology Co., Ltd.; cat. no. D8230) for color detection. The tissue sections were then counterstained with hematoxylin. The negative control sections were obtained by omitting the primary antibody or by using an unrelated rabbit polyclonal antibody.

The antigen levels in the IHC stained tissue samples were evaluated in 10 random fields (400x magnification) for each section. A total of 100 cells/field were categorized as follows: 0, 0-5 cells were stained; 1, 6-25 cells were stained; 2, 26-50 cells stained; 3, 51-75 cells stained; and 4, 76-100 cells stained. In addition, the staining intensity was scored as follows: 0, no staining; 1, weak staining; 2, moderate staining; 3, intense staining. The intensity score was multiplied by the frequency score, in order to obtain the final score. A final score of ≥6 indicated high expression levels, whereas a score of <6 indicated low expression levels (45).

Western blotting analysis of IRE1β. Protein lysates were prepared from collected tissue samples in radioimmunoprecipitation assay lysis buffer (Solarbio Science and Technology Co., Ltd.) on ice by homogenization with a grinder. The supernatant was obtained following centrifugation at 10,800 x *g* for 15 min at 4˚C. A bicinchoninic acid assay (Solarbio Science and Technology Co., Ltd.,) method was used to determine the protein concentrations. Protein (30 μg) from each tissue sample was denatured and resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Following blocking for 1 h at 37˚C in 5% skim milk, the membranes were incubated with the anti-IRE1β antibody (Abcam; dilution 1:200; cat. no. ab135795) for 3 h at 37˚C, then washed four times in 1X TBST. The membranes were subsequently incubated with HRP-conjugated anti-IgG secondary antibody (Boster Biological Technology Co., Ltd.; dilution, 1:1,000; cat. no. BA1054) and then washed four times in 1X TBST. The proteins were visualized using an enhanced chemiluminescence reagent (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. An anti-β-actin mouse monoclonal antibody (Abcam; dilution, 1:3,000; cat. no. ab8226) was used to normalize for the protein loading. The secondary antibody for β-actin was a HRP-conjugated goat anti-mouse IgG (Boster Biological Technology Co., Ltd.; dilution, 1:1,000; cat. no. BA1050). ChemiDoc XRS (Bio-Rad Laboratories, Inc.) was used to capture the images, and the intensity of the images was quantified using ImageJ software v1.48 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. A Student's *t*-test and a Mann-Whitney U-test were used to determined significant differences between the groups. A Wilcoxon signed-rank test was used for non-parametric data. Spearman's bivariate analysis was used to determine the correlation between IRE1β and MUC2 mRNA expression levels. The data are presented as the mean ± standard deviation. All statistical analysis was performed using using the SPSS 19.0 statistical package (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant result.

Results

Clinical characteristics. A total of 42 patients were recruited for the current study and the clinical features are summarized in Table I. The ages of the patients ranged from 44-82 years (average, 61.3 years). A total of 18 patients were male and 24 were female. The discrepancy in gender ratio of this study from the China colorectal report may be due to the gender ratio in the local area (4,46).

Expression levels of IRE1α, XBP1, IRE1β and MUC2 mRNA in CRC tissues. XBP1 expression levels are increased in numerous types of cancer, including CRC (8,17,18). During UPR, IRE1α initiates the splicing of XBP1u mRNA to XBP1s, generating an XBP1 transcription factor that regulates a subset of UPR genes to constitute the IRE1α-XBP1 signaling pathway (8,16). RT-qPCR was used to analyze the mRNA expression levels of IRE1α, XBP1u and XBP1s in tissue samples from patients with CRC. The paired colon tissue samples were analyzed in 31 patients for IRE1α mRNA and 12 patients for XBP1u and XBP1 s mRNAs. It was identified that IRE1α, XBP1u and XBP1s mRNAs were expressed at similar levels in the CRC and non-cancerous tissues (Fig. 1A and Table III).

IRE1β, an analog of IRE1α, is specifically expressed in the epithelium of the gastrointestinal and respiratory tracts;

Table I. Clinicopathological characteristics of the included patients with colorectal carcinoma (n=42).

Variable	Number of cases (%)
Gender	
Male	18 (42.9)
Female	24 (57.1)
Age (years)	
<60	18 (42.9)
≥60	24 (57.1)
Tumor size (cm)	
<5	11 (26.2)
≥5	28 (66.7)
Data incomplete	3 (7.1)
Differentiation	
WMDC	11 (26.2)
MDC	27 (77.1)
PMDC	4 (9.5)
Lymphatic node metastasis	
Negative	15 (35.7)
Positive	27 (64.3)
TNM stage	
I-II	24 (57.1)
III-IV	18 (42.9)

WMDC, well-moderately differentiated carcinoma; MDC, moderately differentiated carcinoma; PMDC, poor-moderately differentiated carcinoma; TNM, tumor node metastasis.

MUC2 expression in the intestine is regulated by IRE1β, but not by IRE1α (27,47). IRE1β and MUC2 mRNA expression levels were evaluated in 35 patients with CRC. The mRNA expression levels of IRE1β and MUC2 in the cancerous tissues were 2.1 and 4.5-fold lower, respectively, compared with in the adjacent non-cancerous colon mucosa (Fig. 1B and 1C). It was identified that MUC2 mRNA expression levels were positively correlated with IRE1β mRNA expression levels (r=0.45; P=0.01; Fig. 1D).

mRNA expression levels of IRE1β, but not IRE1α or MUC2, were associated with lower clinical stages, metastasis and poor differentiation in CRC. To evaluate the clinical significance of changes in the mRNA expression levels of IRE1β, IRE1α and MUC2 in CRC tissues, the association between the mRNA expression levels of these genes and the clinicopathological features of the patients with CRC, were analyzed. It was identified that IRE1β mRNA expression levels were significantly associated with tumor differentiation (P=0.049), lymph node metastasis (P=0.043) and TNM stage (P=0.018) (Table IV), but not with gender (P=0.709), age (P=0.558) and T-stage classification (P=0.384) (48,49) (data not presented). Although the mRNA expression levels of IRE1β in the tumor tissues were low compared with the adjacent normal tissues, in poor-moderately differentiated CRC tissues the IRE1β mRNA expression levels were high as compared with in well-differentiated CRC tissues. Furthermore, those patients with lymphatic metastasis or stage III-IV CRC, had high IRE1β mRNA expression levels, as compared with those patients without lymphatic metastasis or stage I-II CRC. IRE1α and MUC2 mRNA expression levels were not observed to be significantly associated with patient clinicopathological features (Table IV).

Immunohistochemistry of IRE1α, IRE1β and MUC2 in CRC tissues. ER stress is emerging as an important factor in tumor pathogenesis (8,9). However, to the best of our knowledge, there are no previous reports on the role of IRE1α in the tumorigenesis of CRC. Although it was demonstrated in the current study that the mRNA expression levels of IRE1α are similar in CRC and adjacent normal tissues, as IRE1α regulates ER stress at the protein level, the IRE1α protein expression levels were also evaluated using IHC (Fig. 2A and B). IRE1α was expressed in the plasma membrane of non-cancerous colon epithelium and IRE1α was stained at apical surface of cancerous epithelial cells in the colon. In submucosa, certain unidentified cells also had positive nuclei staining for IRE1α. Again, there was no significant difference in IRE1α protein expression levels between CRC and non-cancerous tissue (Fig. 2C).

Aberrant mucin accumulation in the goblet cells of mouse colons was observed when the IRE1β gene was deleted (33), and IRE1β was revealed to be required for airway mucin excretion (27). In the current study, it was also identified that the IRE1β protein was expressed in colon epithelial cells, including in goblet cells. However, by contrast with a prior animal study (33), the results did not demonstrate a predominant IRE1β-positive staining in human colon goblet cells. Above the nuclei of the epithelial cells, a strong positive staining was observed in non-cancerous tissue samples (Fig. 2D). In the cytoplasm of CRC tissues, IRE1β positive staining was comparatively low (Fig. 2E and F); in normal and cancerous tissues, there were unidentified cells that were weakly stained in the submucosa. Similar to the mRNA expression of IRE1β, the downregulation of IRE1β was significantly associated with tumor differentiation (P=0.047), lymph node metastasis (P=0.009) and TNM stage (P=0.001) (Table V). No significant association was identified between IRE1β expression levels and other clinicopathological factors, including gender (P=0.998), age (P=0.115) and tumor size (P=0.742) (data not presented).

MUC2 protein is present in the goblet cells of the colon epithelium (34,50). It has been reported that the IHC staining of MUC2 in goblet cells exhibits whole-cell filled or peri-nuclear staining patterns (51,52). In the present study, the MUC2 staining pattern was half-filled in the goblet cells, and MUC2 expression levels were significantly decreased in these CRC tissues (P<0.001; Fig. 2G-I). Similar to MUC2 mRNA expression levels, the MUC2 protein expression levels, as quantified by IHC staining, were not significantly associated with the clinicopathological factors of patients with CRC (data not presented).

Western blot analysis of IRE1β expression in CRC tissues. The protein expression levels of IRE1β in 13 paired CRC and adjacent normal tissue samples were evaluated using western blotting. Similar to the expression levels of IRE1β in the mRNA and IHC results, a significant difference was identified

Table II. Primers sequences for reverse transcription-quantitative polymerase chain reaction.

mRNA	Gene	Primer sequence (5'-3')	Amplicon (bp)
NM-001433	IRE1α	Forward CTCCGAGCCATGAGAAATAAG Reverse GGGAAGCGAGATGTGAAGTAG	113
NM-001079539	XBP1s	Forward AAGTGGTAGATTTAGAAGAAGAGAA Reverse ACCTGCTGCGGACTCAG	200
NM-005080	XBP1u	Forward AGTCCGCAGCACTCAG Reverse GGGTCCAAGTTGTCCAGA	150
NM-033266	IRE1β	Forward TCCCCTTATAGGACCGGAAC Reverse GTGACTGGCTGGAGAAGGAG	147
NM-002457	MUC2	Forward GACACCATCTACCTCACCCG Reverse TGTAGGCATCGCTCTTCTCA	103
NM-00110	β-actin	Forward AGCACTGTGTTGGCGTACAG Reverse CTCTTCCAGCCTTCCTTCCT	116

IRE1, inositol-requiring enzyme 1; MUC2, mucin 2; XBP1, X-box binding protein 1; s, spliced; u, unspliced; bp, base pairs.

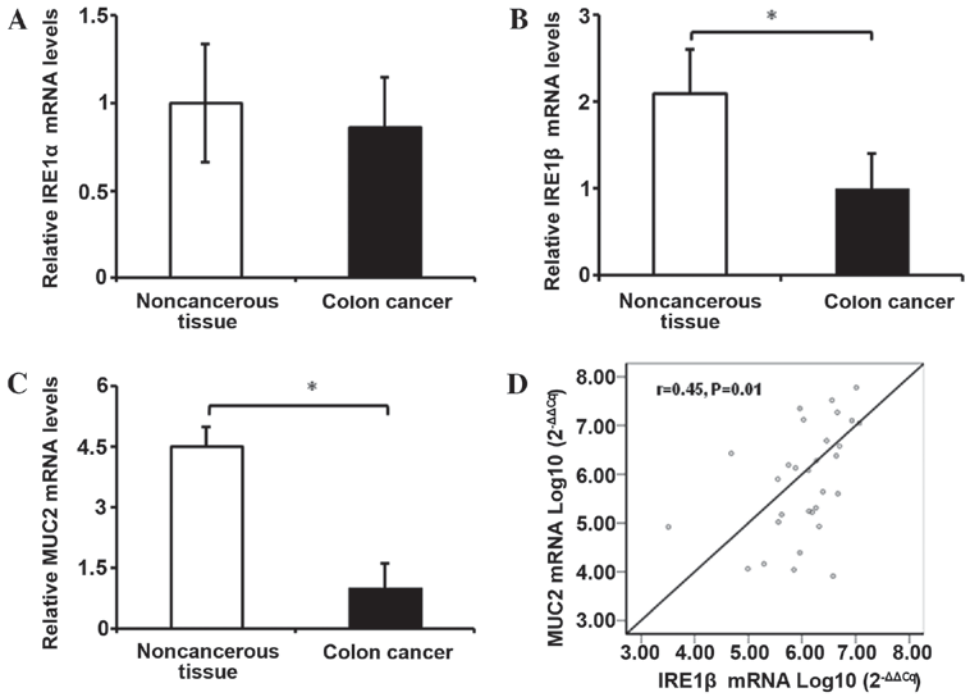

Figure 1. IRE1α, IRE1β and MUC2 mRNA expression in CRC tissues. (A) The mRNA expression levels of IRE1α in cancerous tissues were similar to those in paired noncancerous tissues (n=31). (B and C) IRE1β and MUC2 mRNA expression levels were decreased in CRC tissues, compared with paired non-cancerous tissues (n=35), and (D) MUC2 mRNA expression levels were positively associated with IRE1β mRNA expression levels. The data are presented as the mean ± standard deviation. *P<0.05, vs. the non-cancerous tissues. IRE1, inositol-requiring enzyme 1; MUC2, mucin 2; CRC, colorectal cancer.

in the IRE1β protein expression levels, which were 8-fold lower in cancerous tissues, compared with the adjacent normal control tissues (Fig. 3A and B; P<0.001), which indicated that the expression level was decreased in CRC tissues at the transcriptional and translational levels.

Discussion

ER stress affects tumorigenesis and elevation of the XBP1 transcription factor has previously been reported in numerous types of cancer, including CRC (9,19,21). As XBP1 mRNA is processed by IRE1α, the IRE1α, XBP1u and XBP1s mRNA expression levels, and IRE1α protein expression levels, were analysed in cancerous and adjacent normal colon tissue samples; however, no significant difference was observed between the two tissues. Although Fujimoto *et al* (21) identified that XBP1 gene and protein expression levels were increased in 4/5 CRC tissues, the sample size in their study was small. In the current study, XBP1u and XBP1s gene expression levels were analyzed in 12 cases of CRC. Additionally, the IRE1α mRNA and protein expression levels were evaluated and no significant changes were observed in CRC

Table III. XBP1u and XBP1s mRNA expression levels in patients with colorectal cancer.

No.	Patient	Gender	Age (years)	Differentiation	Ratio (C/N)	
					XBP1u	XBP1s
1	C58	m	44	PDC	2.5	0.8
2	C57	f	65	MDC	1.0	1.0
3	C24	f	53	MDC	0.8	0.8
4	C29	m	64	MDC	1.1	1.0
5	C31	m	61	MDC	0.9	0.8
6	C33	m	61	MDC	2.0	2.5
7	C52	f	61	MDC	1.1	0.8
8	C53	f	60	MDC	0.5	0.4
9	C54	f	52	WMDC	1.4	2.5
10	C55	f	68	WDC	1.1	1.3
11	C56	f	47	WDC	5.0	5.0
12	C59	f	47	WDC	1.0	1.4
Mean ± standard deviation					1.1±0.5	1.0±0.6

XBP1, X-box binding protein 1; s, spliced; u, unspliced; f, female; m, male; PDC, poorly differentiated carcinoma; MDC, moderately differentiated carcinoma; WMDC, well-moderately differentiated carcinoma; WDC, well-differentiated carcinoma; C/N, cancerous tissue/noncancerous tissue.

Table IV. IRE1α, IRE1β and MUC2 mRNA expression levels in patients with CRC.

Variables	IRE1α mRNA			IRE1β mRNA			MUC2 mRNA		
	n	Ratio of C/N	P-value	n	Ratio of C/N	P-value	n	Ratio of C/N	P-value
Total patients	31			35			35		
Clinical stage			0.328			0.018[a]			0.355
I+II	17	1.0±0.1		21	3.7±0.6		20	1.0±0.4	
III+IV	14	1.3±0.2		14	1.0±0.3		15	2.4±1.2	
LN metastasis			0.135			0.043			0.692
Yes	12	1.5±0.1		11	3.3±0.5		13	1.5±0.7	
No	19	1.0±0.1		24	1.0±0.3		22	1.0±0.4	
Differentiation			0.605			0.049[a]			0.519
Well	9	1.1±0.1		8	1.0±0.3		10	1.0±0.5	
Moderate or Poor	22	1.0±0.1		27	4.9±0.9		25	2.0±0.9	

[a]Data showed heterogeneity of variance. Student's *t*-tests and Mann-Whitney U tests were used to determine the significance of differences between groups. LN, lymph node; C/N, cancerous tissue/non-cancerous tissue; IRE1, inositol-requiring enzyme 1; MUC2, mucin 2. The data are presented as the mean ± standard deviation.

tissues, compared with the adjacent non-cancerous tissues. Therefore, the results suggest that the IRE1α-XBP1 signaling pathway does not have an important role in the progression of CRC.

Colon epithelial cells also express IRE1β, an analog of the ubiquitous IRE1α, which has differing functions to IRE1β with regard to cell survival and apoptosis (29-32,47). IRE1β is inefficient at cleaving XBP1 mRNA and directly interacts with unfolded proteins in the ER by association with glucose-regulated protein 78, which is crucial regulator of ER stress (26). Therefore, the IRE1β expression levels in patients with CRC were also analyzed. The present study demonstrated that the mRNA and protein expression levels of IRE1β were significantly decreased in CRC tissues. It is possible that the decreased expression levels reflect the transition of normal epithelial cells to cancerous cells. The IHC results revealed positive IRE1β staining in all epithelial cells, suggesting that IRE1β affects not only goblet cells, but also epithelial cells. A previous study reported that IRE1β regulates lipid absorption by mediating the transcription of

Table V. Association between the immunohistochemical staining of IRE1β and the clinical characteristics of patients with colorectal carcinoma (n=35).

Variable	Patients, n	IRE1β		P-value
		Low, n	High, n	
Clinical stage				0.001
I+II	21	17	4	
III+IV	14	2	12	
Lymph node metastasis				0.009
Yes	11	2	9	
No1	24	17	7	
Pathologic differentiation				0.047
Well	8	7	1	
Moderate or Poor	27	12	15	

Differences were analyzed by χ^2 test. IRE1, inositol-requiring enzyme 1.

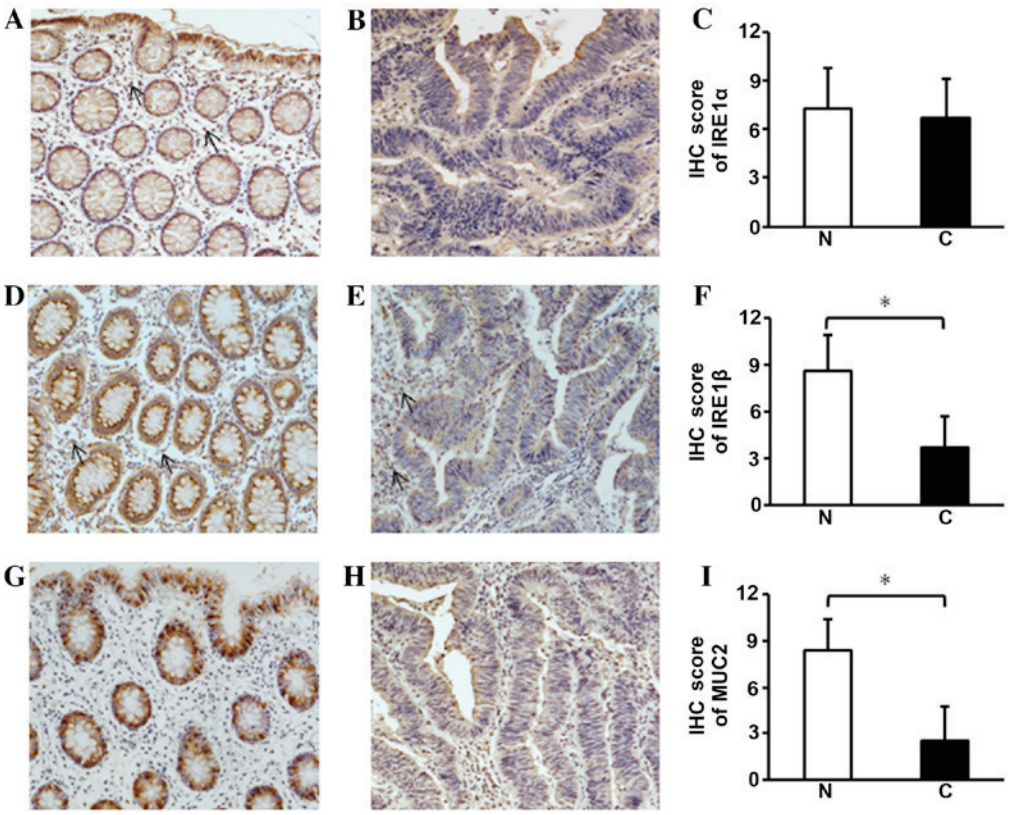

Figure 2. Immunohistochemistry of IRE1α, IRE1β and MUC2 in CRC tissues. (A) IRE1α was expressed in the cytoplasm of epithelial cells in the crypts surface of non-cancerous tissues. In the submucosa unidentified cells were observed to have weakly positive staining (arrow). (B and C) In CRC tissues there was a similar level of the IRE1α protein; (D) IRE1β was observed in the cytoplasm above the nuclei of the non-cancerous colonic epithelial cells. (E and F) In the CRC tissues, the cytoplasmic staining for IRE1β was faint, suggesting the expression of IRE1β was decreased; in the submucosa of the non-cancerous tissues and cancerous tissues there were also unidentified cells with weakly positive staining (arrow). (G) The staining for MUC2 was intensely positive in the goblet cells of the non-cancerous colonic epithelium; (H and I) in the CRC tissues MUC2 staining was barely visible. Representative immunostaining for (A and B) IRE1α, (D and E) IRE1β and (G and H) MUC2 and the semi-quantification of (C) IRE1α, (F) IRE1β and (I) MUC2 for each tissue group. The data are presented as the mean ± standard deviation (n=35 for all three groups). *P<0.05, vs. the non-cancerous tissues. N, noncancerous tissues; C, cancerous tissues; IRE1, inositol-requiring enzyme 1; MUC2, mucin 2; CRC, colorectal cancer; IHC, immunohistochemistry.

microsomal triacylglycerol transfer protein expression in the colon epithelium (53). However, the association between the change in lipid absorption and the tumorigenesis of CRC requires further study for elucidation. It was hypothesized that decreased IRE1β expression levels may be associated with tumorigenesis, as IRE1β is a protective factor for colitis

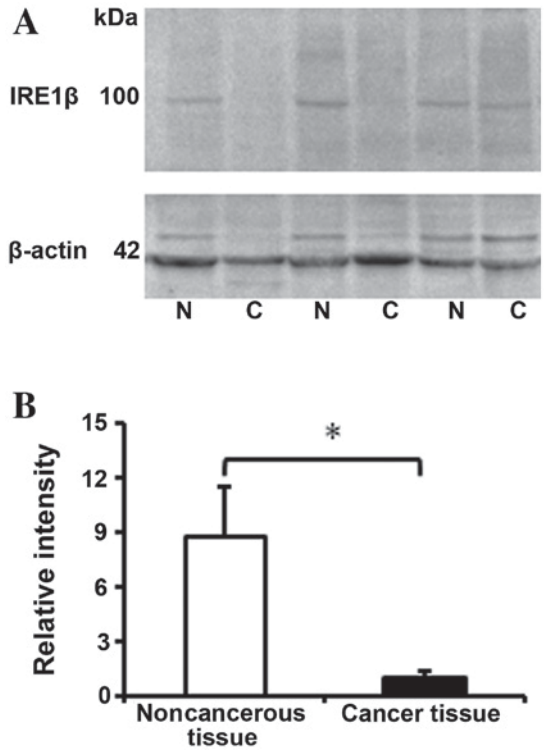

Figure 3. Western blot analysis of IRE1β. (A) The representative immunoblotting images correspond to IRE1β protein expression levels in CRC tissues. (B) The protein expression levels of IRE1β were downregulated in CRC tissues, compared with non-cancerous tissues. The data are presented as the mean ± standard deviation (n=13). *P<0.05, vs. the non-cancerous tissues. N, noncancerous tissues; C, cancerous tissues; IRE1, inositol-requiring enzyme 1; CRC, colorectal cancer.

and is involved in cell apoptosis (26,32). Intestinal inflammation and cell apoptosis are putatively associated with occurrence of CRC (54). Analysis of the mRNA expression levels of IRE1β in tumor tissues revealed that higher IRE1β mRNA expression levels were significantly associated with poor tumor differentiation, lymph node metastasis and later TNM stage. The results suggest that IRE1β may be involved in the development of CRC. The results of the present study are concordant with the *in vitro* study conducted by Dai *et al* (55), who identified that the mRNA expression levels of IRE1β were high in undifferentiated Caco-2 cells, and were correspondingly decreased following the differentiation of these cells (55).

Two previous studies have reported that IRE1β is an important regulator of MUC2 secretion (28,33). In animal model studies, IRE1β$^{-/-}$ mice were more susceptible to dextran sodium sulfate (DSS) -induced colitis, compared with wild type mice (26). The loss of intestinal mucin also increases the sensitivity of mice to DSS-induced colitis (56). IRE1β is essential for UPR in goblet cells, and MUC2 is its target protein (27,33). In the current study, MUC2 expression levels were revealed to be decreased in colorectal adenocarcinomas. The results are concordant with previous studies, in which nonmucinous CRC tissues were negative for MUC2 expression (57,58). This may be due to a decreased number of goblet cells in non-mucinous CRC tissues, as the expression of MUC2 was positively correlated with the mRNA expression levels of IRE1β. However, it has previously been revealed that the methylation of the MUC2 promoter and the loss of functional tumor protein 53 may decrease MUC2 expression levels in CRC tissues (59,60). Correlation analysis in the present study indicated that the expression levels of MUC2 were positively associated with the mRNA expression levels of IRE1β. IRE1β and MUC2 act as protective factors to maintain the intestinal physiological homeostasis, and the suppression of the two proteins may be associated with the tumorigenesis of CRC.

In conclusion, the results of the current study revealed that the decreased expression levels of IRE1β in CRC tissues were associated with clinical features of patients with CRC. IRE1β gene expression levels were positively correlated with those of MUC2, indicating that IRE1β and MUC2 may be involved in the tumorigenesis of CRC. The association of IRE1β and MUC2, as well as the significance of IRE1β in CRC, require further studies in order to identify novel therapeutic targets for this type of cancer.

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