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Original Article

Expression and Clinical Significance of IRE1-XBP1s, p62, and Caspase-3 in Colorectal Cancer Patients

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Abstract

Background: Three main cell signaling pathways including the endoplasmic reticulum stress (ERS) response, autophagy, and apoptosis play critical roles in both cell survival and death. They were found to crosstalk with one another during tumorigenesis and cancer progression. This study aimed to investigate the expression of the spliced form of X-box binding protein 1 (XBP1s), p62, and caspase-3, as the essential biomarkers of ERS, autophagy, and apoptosis in patients with colorectal cancer (CRC), as well as the correlation between their expression and clinicopathological data.

Methods: This retrospective study was conducted on formalin-fixed paraffin-embedded (FFPE) blocks, which were collected from patients and their tumor margins, from the tumor bank of Imam Khomeini Hospital (Tehran, Iran) from 2017 to 2019. Tissue microarray (TMA) was used to measure the XBP1s, p62, and caspase-3 biomarkers. Data were analyzed using SPSS software version 20, and $P \leq 0.05$ was considered statistically significant.

Results: Evaluating the total of 91 patients, a significant relationship was found between XBP1s expression and TNM stage (P=0.003), primary tumor (pT) (P=0.054), and the degree of differentiation (P=0.006); and between caspase-3 with pT (P=0.004), and lymphovascular invasion (P=0.02). However, no significant correlation was found between p62 and clinicopathological data. Furthermore, a positive relationship between XBP1s and p62 was confirmed (correlation coefficient: 22.2% and P=0.05).

Conclusion: Our findings indicated that XBP1s could be considered as a target for therapy in personalized medicine.

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Keywords ● Apoptosis ● Autophagy ● Caspase-3 ● Colorectal neoplasm ● Endoplasmic reticulum stress

What's Known

- The vital mechanism mediating cell survival and death include endoplasmic reticulum stress (ERS) response, autophagy, and apoptosis.
- In cancer patients, XBP1s, p62, and caspase-3 are known as critical biomarkers of ERS, autophagy, and apoptosis.

What's New

- There was a significant correlation between the clinical stages and the expression of XBP1s in tumor tissues.
- XBP1s have the potential to be considered as a targeted treatment for CRC tumors.

Introduction

Colorectal cancer (CRC) is one of the most prevalent cancers of the gastrointestinal tract and is known as the third cause of mortality worldwide.[1] Despite the success in extending overall survival with various treatments, CRC remains a challenge.[2, 3] Finding new targets for efficient treatment has always been a concern.[4] Extensive research on the Endoplasmic reticulum stress (ERS) response and autophagy pathways has confirmed their role in the occurrence and progression of several malignancies, including CRC.[3, 5, 6]

ERS and unfolded protein response (UPR) are caused by different pathological or physiological conditions, including nutrient deficiency, hypoxia, and accumulation of misfolded proteins in the lumen of the endoplasmic reticulum (ER). Following UPR activation, the chaperone of ER, termed glucose-regulated protein 78 (GRP78), dissociates from the ER sensors, which are known as protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1α (IRE1α), and activating transcription factor 6 (ATF6). Each of the three sensors has its own distinct pathway; however, they exhibit crosstalk and are closely related to CRC development.[7] Due to the increased protein folding caused by ERS, the cells initially adapt and survive, while under prolonged or extreme ERS, the cells enter the apoptosis phase.[8] Among three sensors, IRE1 is the most conserved UPR-related arm, which plays critical roles in both tumor survival and apoptosis.[9] The N-terminal region of IRE1 binds to unfolded proteins, whereas the C-terminal region in the cytoplasm has two kinase and RNase domains. Upon activation of UPR, IRE1 becomes autophosphorylated and splices the X-box binding protein 1 (XBP1s) mRNA with its RNase domain, resulting in the separation of the 26-nucleotide of its intron. The spliced XBP1s regulates the expression of the genes involved in protein folding, protein secretion, the endoplasmic reticulum-associated protein degradation system (ERAD), and lipid metabolism by acting as a transcription factor and transferring them to the nucleus.[10] Increased IRE1/XBP1s activity was observed in various malignancies, and also its association with tumorigenicity, cancer progression, and poor survival was confirmed.[8, 11]

Autophagy is a conserved catabolic pathway in cells that is triggered in response to various stress conditions, such as organelle damage and nutritional deficiency, and is regulated by autophagy-regulated genes (ARG). In this pathway, autophagosomes are formed by cytoplasmic dabble-membrane, which could engulf damaged organelles or unrequited macromolecules. The autophagosomes are finally fused with the lysosome to degrade their substances.[12] In each step, different autophagy genes are involved and can be detected as indicator markers. Considering the importance of autophagy and its role in various diseases, the investigation of its markers should be conducted more cautiously. p62, which is also known as sequestosome1 (SQSTM1), is one of the most essential autophagy markers. p62 is involved in the degradation of cargo and is eliminated during the autophagy process. Indeed, autophagy induction leads to a decreased level of p62, whereas autophagy inhibition accelerates its accumulation. One of the techniques to monitor autophagy is to measure p62 degradation.[13] Autophagy plays a dual role in tumor initiation and cancer progression. Indeed, under normal conditions, autophagy maintains cell stability by removing damaged proteins and organelles that are not toxic. However, autophagy indicates a different behavioral pattern during the development of tumors, provides nutrients for cancerous cells, and induces tumor growth.[12] Therefore, induction or inhibition of autophagy in different stages of cancer progression is critical for treatment, particularly in CRC.[14] However, the correlation between autophagy genes and CRC clinicopathological data requires further study.

Apoptosis is another crucial mechanism determining cell destiny. Apoptotic pathways, which are mediated under tightly controlled pathological or physiological cell conditions, were employed as a targeted therapy for CRC.[15] Its activation in tumors is critical for disease progression.[16] Apoptosis is primarily activated by two distinct signaling pathways, including intrinsic (mitochondrial-mediated) and extrinsic pathways.[17] Both pathways trigger caspase-3 activation, which leads to cell death. It is a significant mediator of apoptosis that is activated when the cell is exposed to cytotoxic drugs, or different therapies such as radiotherapy, or immunotherapy. The majority of anticancer therapies are designed to activate caspase-3, which is a frequently used marker to assess cancer therapy efficiency. Caspases-3, however, has non-apoptotic functions in the cell, such as tumor angiogenesis and tumor relapse.[18] Nevertheless, evaluating caspase-3 activation as a diagnostic marker is mainly used to evaluate the efficacy of cancer treatment.[16, 19]

The present study aimed to determine the expression levels of the most important mediators of UPR (XBP1s), autophagy (p62), and apoptosis (caspase-3) pathways in different stages of CRC and to correlate these markers with clinicopathological data of CRC.

Patients and Methods

Patients

This retrospective study was conducted from 2017 to 2019. The formalin-fixed paraffin-embedded (FFPE) CRC blocks and their tumor margin samples were selected from the archives of the tumor bank of Imam Khomeini Hospital affiliated with Tehran University of Medical Sciences (Tehran, Iran). The study was

approved by the Ethics Committee of Tehran University of Medical Sciences (IR.NIMAD.REC.1398.265). Based on the Cochran's formula, the sample size was calculated as 108. However, based on our criteria and the quality of FFPE, a total of 91 samples were collected. In this research, all of the colorectal adenocarcinomas were included. However, signet-ring cell, mucinous, adenosquamous, squamous, and undifferentiated CRC carcinoma were excluded.

The clinicopathological data were listed in reference to each patient's pathology reports. Regarding the College of American Pathologists (CAP) and approval of 2020 (Protocol for the Examination of Excisional Biopsy Specimens from Patients with Primary Carcinoma of the Colon and Rectum), all the available pathological findings were recorded as follows: 1- Tumor sites are classified as colon, rectum, and recto-sigmoid. 2- Adenocarcinoma was the only histological type of tumor that was recorded, the others were excluded. 3- Histologic grades are categorized as G1, G2, and G3, which are well-differentiated, moderately differentiated, and poorly differentiated, respectively. 4- Tumor metastasis (M) is classified as either positive (tumor invades) or negative. 5- Positive and negative lymphovascular invasions were documented. 6- Positive and negative perineural invasions were also reported. 7- Tumor size is divided into three categories: less than 5 cm, 5 cm, and more than 5 cm. 8- The primary tumor (pT) is classified as pT1 (invades the submucosa), pT2 (invades the muscularis propria), pT3 (invades the peri colorectal tissues through the muscularis propria), and pT4 (invades the visceral peritoneum or invades or adheres to the adjacent organ or structure). 9- Neoadjuvant therapy and treatment effects are listed as positive and negative. 10- TNM stage was recorded for stages 1, 2, 3, and 4, respectively. Lymph node metastasis was recorded to PN0 (not yet disseminated to surrounding lymph nodes), PN1 (spread to one to three nearby lymph nodes), and PN2 (spread to four or more nearby lymph nodes).

Tissue Microarray

The block arrays were designed to characterize immunohistochemically protein expression using three distinct markers (XBP1s, p62, and caspase-3). Briefly, 1.5-mm-diameter cylinders of each CRC tissue and their margins were taken from the defined areas of each paraffin block and placed into a new block, with one paraffin block for each biomarker prepared from all tissue blocks.

Immunohistochemistry Assay

The cut segments from previously prepared tissue microarray blocks were transferred to IHC special slides. Then, the slides were placed in a bain-marie at 56 °C for 24 hours. As previously stated,[20] antigen retrieval was performed using the antibodies (XBP1s, Cat. No. 647501, BioLegend, Inc., USA, p62/ SQSTM1 [D-3]: sc-28359, Santa Cruz Biotechnology, Inc., USA, and caspase-3, Santa Cruz Biotechnology, Inc., USA) after washing with serial dilutions of ethanol and phosphate-buffer saline. The markers had the following patterns: XBP1s and caspase-3 were cytoplasmic, and p62 was both cytoplasmic and nuclear. Additionally, a standard protocol was followed while performing hematoxylin and eosin (H&E) staining.[21]

Interpretation of Tissue Immunohistochemical Reactions

The IHC staining uses the Allred scoring system as a way to convert qualitative information into quantitative data. The two parameters that make up this system are proportion and intensity scoring. The proportion score is shown by the following numbers: 0 for no visible cells, 1 for less than 1% of detectable cells, 2 for 1- 10%, 3 for 11-33%, 4 for 34-66%, and 5 for 67-100% cell staining. The intensity rating is based on the color intensity and ranges from 0 to 3. In this scoring, 0 stands for negative staining, whereas a score of 1 to 3 denotes weak, intermediate, and strong staining, respectively.

Then, two parameters were added together and reported as follows: 0-1 indicates a negative effect, 2-3 suggests a mild effect, 4-6 indicates a moderate effect, and 7-8 denotes a strong effect. The final report was based on 10 high power fields (HPF) of microscopic vision.[22] The same markers were also used to evaluate the normal tissues. To correlate the pathological data with the IHC scores, we classified them as negative, mild, moderate, and strong staining.[23]

Statistical Analysis

The clinical data and IHC scoring were analyzed using SPSS software version 20 (SPSS Inc., Chicago, IL, USA). The qualitative variables were measured using the Chi square and Fisher's exact tests. The Spearman's correlation coefficient test was used to measure the degree of relationship between XBP1s, p62, and caspase-3. In all experiments, $P \leq 0.05$ was considered statistically significant.

Results

Clinical Characteristics

All 91 patients were diagnosed with CRC,

and all of their clinicopathological data were recorded. Of these patients, 36.3% were female, and 63.7% were male. Their ages ranged from 23 to 93 years old at the time of diagnosis (mean=58.6 years). The colon was the site of the majority of the tumors (80.2%), followed by the rectum (13.2%), and the rectosigmoid (6.6%). Histologically, all of the tumors were adenocarcinoma (100%). The classification of tumors according to primary tumor (pT) classification was 8.8%, 23.1%, 38.5%, and 29.7% in pT1 to pT4, respectively. The differentiation status was scored as well-differentiated (20.9%), moderately differentiated (61.5%), and poorly differentiated (12.1%). The frequency of the tumors in the different clinical stages (TNM stage), was 31.9%, 17.6%, 44%, and 3.3%, in stages 1 to 4, respectively.

The Correlation between XBP1s Expression and Clinicopathological Data

To assess the level of ERS activity in a series of CRC patients, XBP1s expression was used as a marker of the IRE1 arm of the UPR. The results showed that XBP1s expression in tumor samples was 57.1% higher than in the different control groups (strong, moderate, and weak staining), indicating that XBP1s and UPR activity were overexpressed during tumor progression (figure 1). Figure 2 shows the IHC staining of these markers in terms of negative and positive staining.

In the next step, the correlation of the XBP1s expression with different clinicopathological data was investigated. The findings revealed no relationship between XBP1s expression and the patient's sex, and the location of the tumors. Although all the tumors recruited in this study were adenocarcinoma, there was no significant correlation between the tumor type and XBP1s expression (P=0.25). Moreover, there was no significant association between XBP1s expression and other pathologic variables such as lymph vascular invasion, perineural invasion, neoadjuvant therapy, treatment effects, lymph node metastasis, and metastases. According to table 1, the only significant correlation was found between XBP1s expression and pT (P=0.054), grade differentiation (P=0.006), and TNM stage (P=0.003). The results showed that XBP1s levels increase as the tumor grows.

XBP1s Expression in Different Stages of CRC

The expression pattern of XBP1s was analyzed in different tumor stages to examine its expression during tumor development. The mean expression of XBP1s was 82.8%, 25%, 47.5%, and 100% in TNM stages 1, 2, 3, and 4, respectively; when the weak, intermediate, and strong staining results were considered positive compared to specimens with negative staining. Figure 3A shows the XBP1s expression level during CRC stages, and figure 3B illustrates the exact percentage of the XBP1s expression in these different TNM stages, which are classified as negative, weak, intermediate, and strong staining. This finding indicated that although the percentage was high in the initial stages of the tumor growth, the expression gradually decreased during tumor progression. Another important factor was the number of samples at different stages.

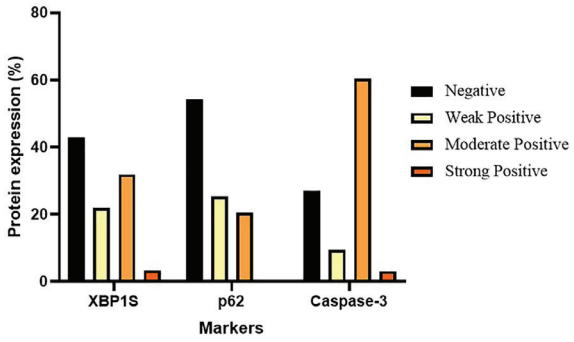

Figure 1: The expression level of protein markers of X-box binding protein 1 (XBP1s), p62, and caspase-3 in colorectal cancer tumors in comparison to their margins were evaluated. The expression of the protein was evaluated by immunohistochemistry (IHC) assay with an Allred scoring system based on negative, weak, intermediate, and strong staining. The weak, intermediate, and staining results were considered positive.

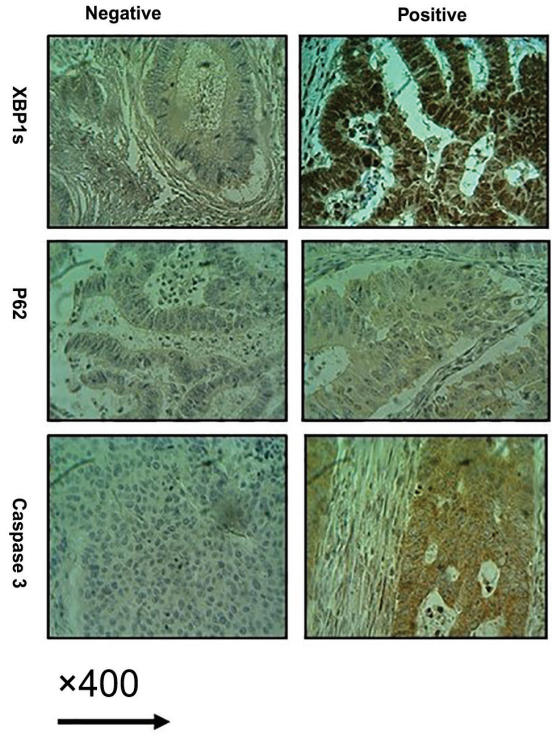

Figure 2: The expression of the X-box binding protein 1 (XBP1s), p62, and caspase-3 markers was evaluated by immunohistochemistry staining method. The proteins of XBP1s, p62, and caspase-3 are more expressed in colorectal cancer (positive) than tumor margin.

Table 1: The correlation between XBP1s expression and clinicopathological data in patients with colorectal cancer (N=91)

Clinicopathological data	Parameter	Frequency (%)	Negative (%)	Weak (%)	Moderate (%)	Strong (%)	P value
Tumor site	Colon	80.2	42.5	23.3	31.5	2.7	0.642
	Rectum	13.2	41.6	16.7	41.7	0	
	Recto-sigmoid	6.6	50	16.6	16.7	16.7	
Histologic Type	Adenocarcinoma	100	43.3	21.1	32.2	3.4	0.25
Primary tumor (pT)	T1	8.8	12.5	50	37.5	0	0.054
	T2	23.1	23.8	28.6	42.8	4.8	
	T3	38.5	42.9	20	34.3	2.8	
	T4	29.6	66.7	11.1	18.5	3.7	
Differentiation grade	Well differentiate	26.4	36.8	31.6	21.1	10.5	0.006
	Moderate differentiate	61.5	35.7	21.4	41.1	1.8	
	Poorly differentiate	12.1	90.9	9.1	0	0	
Lymphovascular invasion	Positive	71.4	46.2	20	29.2	4.6	0.571
	Negative	28.6	34.8	26.1	39.1	0	
Perinueral invasion	Positive	41.8	55.3	21.1	21.1	2.5	0.150
	Negative	58.2	32.6	21.7	41.3	4.4	
Neoadjuvant therapy	Positive	11	60	20	20	0	0.743
	Negative	89	40.7	22.2	33.3	3.8	
Treatment effect	Positive	11	60	20	20	0	0.743
	Negative	89	40.7	22.2	33.3	3.8	
TNM stage	Stage 1	31.9	17.2	31	44.9	6.9	0.003
	Stage 2	17.6	75	12.5	12.5	0	
	Stage 3	44	52.5	17.5	27.5	2.5	
	Stage 4	6.5	0	0	100	0	
Lymph node metastasis	PN0	50.5	37	23.9	34.8	4.3	0.139
	PN1	29.7	37	22.2	40.8	0	
	PN2	19.8	73.3	6.7	13.3	6.7	
Metastasis	Positive	7.7	42.9	14.3	42.8	0	0.913
	Negative	92.3	42.9	22.6	31.0	3.5	
Tumor size	Less than 5 cm	53.8	36.7	28.6	32.7	2	0.212
	5 cm	4.4	50	50	0	0	
	More than 5 cm	41.8	51.4	11.4	31.4	5.8	

Data analysis was performed using the Chi square test and Fisher's exact test. In all experiments, P≤0.05 was considered statistically significant.

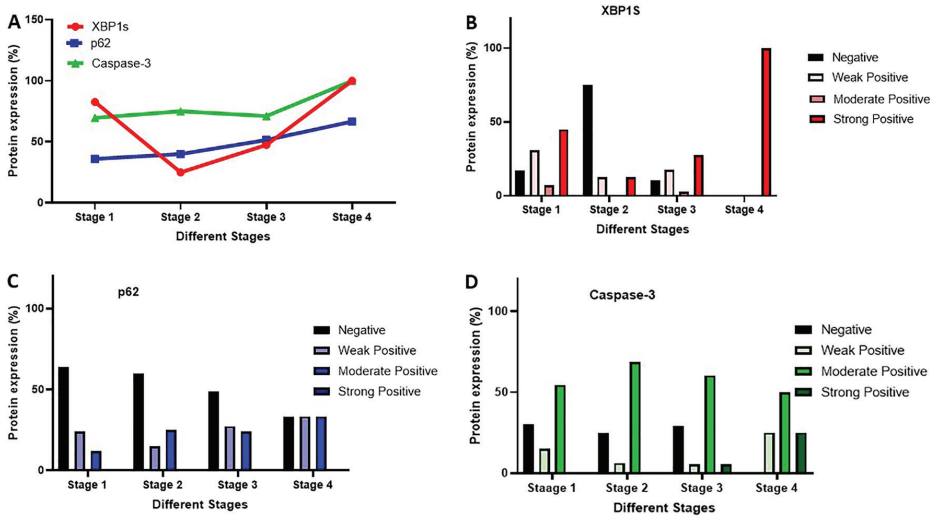

Figure 3: The expression of protein markers during tumor development was evaluated. A) The expression of X-box binding protein 1 (XBP1s), p62, and caspase-3 in different stages of colorectal cancer when weak, intermediate, and strong staining results were considered positive versus negative expression. B) The exact percentage of XBP1s expression in different TNM stages according to negative, weak, intermediate, and strong staining. C) The exact percentage of p62 expression in different TNM stages according to negative, weak, intermediate, and strong staining. D) The exact percentage of caspase-3 expression in different TNM stages according to negative, weak, intermediate, and strong staining

The Correlation between p62 Expression and Clinicopathological Data

To analyze the autophagy activity, the level of p62, which was specifically degraded during autophagy, was assessed. Although p62 expression was lower in CRC tumors than controls (48.55%), the observed differences were not statistically significant. Further investigation of any particular correlation of p62 levels with the clinicopathological data revealed that p62 was not significantly correlated with any clinicopathological variables such as sex, location, tumor type, TNM stage, etc., (table 2).

p62 Expression in Different Stages of CRC

To investigate whether there was a correlation between p62 expression and tumor development, the mean percentage of p62 expression at all the stages was determined. The findings indicated 36%, 40%, 51.5%, and 66.7% expression in stages 1, 2, 3, and 4, respectively; when all weak, intermediate, and strong staining results were recorded positively, in comparison to negatively staining specimens (figure 3A). Figure 3C also illustrates the exact percentage of p62 expression in different TNM stages when classified into negative, weak, intermediate, and strong staining, respectively. The obtained results confirmed that p62 expression levels gradually increased during tumor progression.

The Correlation between Caspase-3 Expression and Clinicopathological Data

To consider apoptosis activity during tumor progression, caspase-3 was analyzed in a series of CRC patients. Caspase-3 expression in CRC tumor samples was 78.95% higher than in the control groups, indicating an increase in the level of apoptotic activity during tumor development.

The correlation between caspase-3 expression and different clinicopathological data was assessed, and no significant correlation was found between caspase-3 expression and sex, location, tumor type, perineural invasion, neoadjuvant therapy, treatment effects, lymph

Table 2: The correlation between p62 expression and clinicopathological data in patients with colorectal cancer (N=91)

Clinicopathological data	Parameter	Frequency (%)	Negative (%)	Weak (%)	Moderate (%)	Strong (%)	P value
Tumor site	Colon	80.2	54.4	23.5	22.1	0	0.852
	Rectum	13.2	54.5	36.44	9.1	0	
	Recto-sigmoid	6.6	50	25	25	0	
Histologic type	Adenocarcinoma	100	53.7	25.6	20.7	0	>0.999
Primary tumor (pT)	T1	8.8	62.5	25	12.5	0	0.839
	T2	23.1	65	25	10	0	
	T3	38.5	48.5	27.3	24.2	0	
	T4	29.6	50	22.7	27.3	0	
Differentiation grade	Well differentiate	26.4	52.9	17.6	29.5	0	0.391
	Moderate differentiate	61.5	55.4	28.5	16.1	0	
	Poorly differentiate	12.1	60	0	40	0	
Lymphovascular invasion	Positive	71.4	52.5	25.5	22	0	0.788
	Negative	28.6	61.9	19.1	19	0	
Perineural invasion	Positive	41.8	70	20	10	0	0.124
	Negative	58.2	47.8	26.1	26.1	0	
Neoadjuvant therapy	Positive	11	70	30	0	0	0.255
	Negative	89	52.1	24.7	23.2	0	
Treatment effect	Positive	11	70	30	0	0	0.255
	Negative	89	52.1	24.7	23.2	0	
TNM stage	Stage 1	31.9	64	24	12	0	0.655
	Stage 2	17.6	60	15	25	0	
	Stage 3	44	48.5	27.3	24.2	0	
	Stage 4	6.5	33.3	33.3	33.4	0	
Lymph node metastasis	PN0	50.5	62.2	20	17.8	0	0.495
	PN1	29.7	45.8	33.3	20.9	0	
	PN2	19.8	50	16.7	33.3	0	
Metastasis	Positive	7.7	50	33.3	16.7	0	0.890
	Negative	92.3	54.5	24.7	20.8	0	
Tumor size	Less than 5 cm	53.8	56.5	26.1	17.4	0	0.928
	5 cm	4.4.	66.7	0	33.3	0	
	More than 5 cm	41.8	54.8	25.8	19.4	0	

Data were analyzed using the Chi square test and Fisher's exact test. In all experiments, P≤0.05 was considered statistically significant.

node metastasis, and metastases. The only significant correlation was observed between caspase-3 expression and the pT (P=0.004), and lymphovascular invasion (P=0.02) (table 3).

Caspase-3 Expression in Different Stages of CRC

The expression pattern of caspase-3 in tumors at different stages was evaluated. In TNM stages 1, 2, 3, and 4, the mean percentages of caspase-3 expression were 69.7%, 75%, 71.1%, and 100% in TNM stages 1, 2, 3, and 4, respectively; when the weak, intermediate, and strong staining results were scored positively compared to negative staining (figure 3A). The percentage of exact caspase-3 expression at different TNM stages was classified according to negative, weak, intermediate, and strong staining. This indicates that the expression of the caspase-3 pattern varied during tumor development, and that caspase-3 expressions increased as the tumor progressed (figure 3D).

The Correlation Analysis between the XBP1s, p62, and Caspase-3 Markers

In the next set of experiments, we investigated pairwise correlations between ERS, autophagy, and apoptosis markers in CRC patients. As presented in table 4, Spearman's correlation analysis between XBP1s and p62 was P=0.05, between XBP1s and caspase-3 was P=0.440, and between p62 and caspase-3 was P=0.784, which reveals a positive correlation between XBP1s and p62 (Correlation Coefficient was 22.2% [P=0.05]). It implies that as the tumor progressed, the level of p62 increased due to increased XBP1s expression. However, there was no significant correlation between XBP1s and caspase-3 levels or between p62 and caspase-3 expression.

Discussion

Our findings indicated that XBP1s and caspase-3 were highly expressed in CRC tumors.

Table 3: The correlation between caspase-3 expression and clinicopathological data in patients with colorectal cancer (N=91)

Clinicopathological data	Parameter	Frequency (%)	Negative (%)	Weak (%)	Moderate (%)	Strong (%)	P value
Tumor site	Colon	80.2	25.3	10.7	60	4	0.845
	Rectum	13.2	23.1	7.7	69.2	0	
	Recto-sigmoid	6.6	50	0	50	0	
Histologic type	Adenocarcinoma	100	27.7	9.6	59.6	3.1	>0.999
Primary tumor (pT)	T1	8.8	0	12.5	87.5	0	0.004
	T2	23.1	30.8	19.2	50	0	
	T3	38.5	43.3	10	46.7	0	
	T4	29.6	15.6	0	75	9.4	
Differentiation grade	Well differentiate	26.4	31.8	13.6	54.6	0	0.689
	Moderate differentiate	61.5	25.9	8.6	60.3	5.2	
	Poorly differentiate	12.1	9.1	9.1	81.8	0	
Lymphovascular invasion	Positive	71.4	26.9	3	67.1	3	0.02
	Negative	28.6	26.9	23.1	50	0	
Perineural invasion	Positive	41.8	27	5.4	64.9	2.7	0.928
	Negative	58.2	26.9	9.6	59.6	3.9	
Neoadjuvant therapy	Positive	11	45.4	9.1	45.5	0	0.533
	Negative	89	24.7	9.4	62.4	3.5	
Treatment effect	Positive	11	45.5	9.1	45.4	0	0.533
	Negative	89	24.7	9.4	62.4	3.5	
TNM stage	Stage 1	31.9	30.3	15.2	54.5	0	0.301
	Stage 2	17.6	25	6.2	68.8	0	
	Stage 3	44	28.9	5.3	60.5	5.3	
	Stage 4	6.5	0	25	50	25	
Lymph node metastasis	PN0	50.5	27.5	11.8	60.7	0	0.269
	PN1	29.7	20.8	12.5	58.3	8.4	
	PN2	19.8	35.3	0	58.8	5.9	
Metastasis	Positive	7.7	0	12.5	75	12.5	0.102
	Negative	92.3	29.5	9.1	59.1	2.3	
Tumor size	Less than 5 cm	53.8	34.8	6.5	58.7	0	0.174
	5 cm	4.4	14.3	28.6	57.1	0	
	More than 5 cm	41.8	20.5	10.3	61.5	7.7	

Data analysis was performed using the Chi square test and Fisher's exact test. In all experiments, P≤0.05 was considered statistically significant.

Table 4: The correlation between XBP1s, p62 and caspase-3 in patient with colorectal cancer (N=91)

Variables	Correlation Coefficient %	P value
XBP1* p62	22.2	0.050
XBP1*Caspase-3	9.2	0.440
p62*Caspase-3	3.3	0.784

This table determines the relationship between the variables two by two that were written in the first column of the table. Data analysis was performed using the Spearman Correlation coefficient test. In all experiments, P≤0.05 was considered statistically significant.

Moreover, there was a significant relationship between XBP1s expression in tumor tissues and TNM stage, pT, and degree of differentiation. A significant correlation was observed between caspase-3 and pT, and lymph vascular invasion. However, no significant correlation was found between p62 and clinicopathological data. In addition, the positive relationship between XBP1s and p62 was confirmed.

Compared to normal tissues or adjacent tumors, the mediators of the ERS, autophagy, and apoptosis were previously found to be upregulated in several malignancies, including breast cancer, melanoma, myeloma, and colorectal tumor tissues.[5, 24-26]

Studies on the patterns of XBP1s expression in different colorectal cancers reported conflicting results. In a study of 12 CRC cases, both the XBP1u and XBP1s gene expression, as well as the IRE1α mRNA, and protein expression levels, revealed no significant differences between the tumor samples and their margins.[27] In comparison to healthy colon epithelial and stromal cells, another experiment showed the XBP1s protein was strongly stained in the cytoplasm of cancer cells.[28] Both studies were conducted with relatively few cases. In contrast, our findings indicated that XBP1s were more expressed in CRC tumors than in tumor margin tissues (57.1%). Although the frequency of tumors is highly serious in all analyses, personalized precision medicine should be taken into consideration while evaluating treatment alternatives or even in screening protocols.[27] Several studies investigated the correlation between XBP1s levels and clinicopathological characteristics in a variety of CRC tumors and reported different findings. For instance, one study found no correlation between XBP1s expression and numerous factors, including age, pathology grade, primary tumor (pT), lymph node metastasis (PN), TNM stage, estrogen and progesterone receptor, HER2 status, or luminal subtype.[11] Another study indicated that XBP1s expression was higher in metastatic and poorly differentiated CRC tissue samples. However, it was lower in moderately- differentiated and well-differentiated tissue samples. Furthermore, a direct correlation was found between the XBP1s immunoreactivity score and tumor invasion.[29] In the present study, we found that poorly differentiated tumors showed higher XBP1s expression than well-differentiated tumors (P=0.006). The overexpression of XBP1s in metastatic and poorly differentiated malignancies confirmed the positive association of XBP1s with increased tumor invasion.[11, 29] In this research, there was no correlation between XBP1s levels and metastasis, which could be due to the small number of metastatic tumors (7.7%). However, XBP1s were highly expressed in 47% of metastatic tumors compared to non-metastatic tumors. Moreover, activation of UPR was associated with drug resistance and tumor recurrence. Previous research reported that there was a relationship between elevated XBP1s expression levels and endocrine-resistant breast cancer cells, which was related to cellular resistance to antiestrogens.[11] In the present study, 60% of the 11 treated patients were negative for XBP1s expression, confirming the effect of treatment on XBP1s. However, further analysis and follow-up are required. Estrogen receptors can induce autophagy and apoptosis, both of which are closely related to tumor development. Gene expression profiles also revealed that there is a correlation between XBP1s expression and different genes involved in cell cycling and apoptosis, such as BCl-2.[30]

Although caspase-3 is a remarkable apoptosis mediator, it is a common biomarker to assess the effectiveness of cancer therapies. However, the non-apoptotic function of caspase-3, such as tumor angiogenesis and tumor recurrence, must be taken into account.[18] Besides, colon cancer cells lacking caspase-3 (CASP3KO) showed reduced tumor invasion and metastasis as well as increased sensitivity to radiotherapy, which suggests that caspase-3 has a potential therapeutic target in colon cancer.[16] Furthermore, TMA analysis of stages 2 and 3 of CRC tumors revealed a correlation between lower levels of active caspase-3 and longer disease-free survival time, particularly in patients receiving 5FU-based adjuvant chemotherapy. Lower active serum caspase-3 levels were also associated with stable disease or tumor regression in patients with metastatic

CRC, indicating that increased caspase-3 activity was associated with a higher risk of recurrence and decreased patient survival.[16]

Although caspase-3 activation induces cell death following chemotherapy, it was shown that tumor resistance to treatment might be due to the stimulation of the proliferation of the tumors' adjacent and non-apoptotic cells. In fact, patients with poor prognoses in many cancer types have highly proliferating tumors.[23]

The findings of the present study indicated that caspase-3 had no relationship with patients' sex, tumor location, tumor types, differentiation grade, invasion, neoadjuvant therapy, treatment effects, TNM stage, lymph node metastasis, tumor metastasis, and tumor size. Caspase-3 was present in CRC tumors at different frequencies depending on the clinical stages (stages 1 to 4 indicated 34.4%, 16.7%, 39.6%, and 4.2%, respectively). Therefore, the expression level was different in the clinical stages. In 88.5% of patients, who experienced a poor response to treatment, 75.5% tested positive for caspase-3, showing a correlation with more ineffective therapies. Furthermore, caspase-3 was shown to be present in all metastatic tumors.

Autophagy markers are also important in cancer prognosis and are widely used to determine cancer progression.[31] Several previous studies reported an association between high p62 expression and poor prognosis in tumors.[32, 33] The findings of the present research indicated that p62, as a useful marker of autophagy, was expressed more in tumor tissue than in adjacent tumors (48.55%). However, there was no significant correlation with other clinicopathological characteristics. However, other studies reported contradictory findings, which may be due to different sample quantities or additional markers, along with p62, which were be examined for autophagy detection. For instance, despite increased expression of p62 in CRC tumor samples, no correlations were found between p62 immunostaining and histological grading, tumor stages, or distant metastasis. Moreover, there was no association between p62 expression and overall survival.[34] Another study reported that although there was no association between staining patterns and pathological features (such as TNM), there was an association between p62 and overall survival. However, the best prognostic marker was found when two markers of LC3B dot-like and p62 dot-like-cytoplasmic staining were highly expressed and also associated with overall survival.[35] Furthermore, previous studies revealed that although there was a correlation between p62 and metastasis in advanced tumors, there was no significant association between p62 and other demographic and clinicopathological variables such as age, sex, tumor size, tumor location, differentiation level, invasion, lymph node metastasis and TNM stage.[36, 37] In the present study, although p62 expression did not correlate with the disease stage, increased expression was observed during tumor progression. Several studies investigated the relationship between different factors of XBP1s with p62. Zhao and others reported a negative correlation between XBP-1u expression and p62 in cancer cells. A stress-related multiprotein known as p62 is essential for the autophagic degradation of ubiquitinated proteins, which, in turn, attenuates ERS by removing the ubiquitinated cargo and decreasing apoptosis.[38] While the present study demonstrated a positive correlation between XBP1s and p62, further studies with larger sample sizes and the other autophagy and ERS markers are required to explain more about the interaction between these signaling pathways.

Although the detection of XBP1s, p62, and caspase-3 markers in tumor specimens has great potential to identify new therapeutic targets, the sample size and patient follow-up are all crucial factors in evaluating markers for targeted therapy in personalized medicine.

Conclusion

As an ERS marker, whose expression rises with tumor development, XBP1s play a role in the pathogenesis of colorectal cancer. Hence, XBP1s have the potential to be considered as a target therapy to treat CRC tumors.

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Authors' Contribution

MK.Z: Data gathering, data analysis, and drafting; M.R: Data gathering, study design, and critical revision; H.M: Data analysis, and critical revision; V.S: Data analysis, and critical revision; MA.M: Data analysis, and critical revision. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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