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The High Expression of SLC7A11 and GPX4 are Significantly Correlated with β-Catenin in Colorectal Cancer

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Background: Existing research shows inducing ferroptosis can improve the effectiveness of tumor treatment. Glutathione peroxidase 4 (GPX4) is a ferroptosis inhibitor. Solute carrier family 7, membrane 11 (SLC7A11) plays a key role in glutathione homeostasis, which is important for protecting cells from oxidative stress. β-catenin is the key protein the Wnt/β-catenin signaling pathway. The purpose of this study was to investigate the expression of SLC7A11 and GPX4 in colorectal cancer (CRC) and their relationship with β-catenin and to analyze the association of these two factors with several clinicopathological features and patient survival.

Methods: This study retrospectively collected paraffin-embedded tissue samples from 120 CRC patients, who received surgical resection between 2017 and 2018. We examined the patterns of expression of SLC7A11, GPX4 and β-catenin by using immunohistochemistry. Analyzing the relationships between SLC7A11, GPX4, β-catenin and clinical pathological parameters and their relationships with overall survival (OS).

Results: Expression of SLC7A11 and GPX4 were high expression in 60.83% and 64.17% among the patients, respectively, and were higher than those in normal tissue. SLC7A11, GPX4 and β-catenin were positively correlated with each other ($P<0.05$). Expression of SLC7A11 and GPX4 significantly correlates with tumor stage and lymph node metastasis ($P < 0.05$). The β-catenin was related to lymph node metastasis, TNM stage and tumor grade. Kaplan–Meier analysis showed that patient's OS in the SLC7A11 and GPX4 were reduced ($P<0.05$). Univariate and multivariate analyses showed that SLC7A11 and GPX4 were independent risk factors for CRC prognosis.

Conclusion: SLC7A11 and GPX4 overexpression is associated with β-catenin and poor prognosis and may be important for predicting CRC invasion, metastasis, and prognosis.

Keywords: colorectal cancer, SLC7A11, GPX4, β-catenin, prognosis

Background

Colorectal cancer (CRC) is one of the most common cancers. According to 2020 statistics, the incidence rate of CRC is 10.0%, ranking third, and the mortality rate is 9.4%, ranking second.[1] Early diagnosis and surgical treatment combined with chemotherapy and radiotherapy will help to improve the clinical outcomes for patients. In recent years, In recent years, with the further understanding of oncology, immunotherapy has become one of the new treatment modalities for CRC.[2,3] Compared with conventional treatment, this treatment mainly uses the patient's own immune system to fight against cancer cells. However, the survival rate of colorectal cancer patients is still low, and one of the important reasons is that tumor cells have developed resistance to the relevant drugs.[4] Therefore, studying molecular markers related to tumor invasion at the molecular level mechanism is of great clinical significance to promote early diagnosis of colorectal cancer, discover new clinical treatment targets and improve patient prognosis.

Ferroptosis is a unique iron-dependent non-apoptotic cell death process that cannot be inhibited by specific inhibitors of regulated cell death (RCD) but can be stopped and reversed by anti-iron oxidants.[5] The cystine/glutamate antiporter SLC7A11 exports intracellular glutamate and imports extracellular cystine at a 1:1 ratio.[6,7] This results in glutathione biosynthesis and antioxidant defense to inhibit ferroptosis, which is essential for protecting cells from oxidative stress. SLC7A11 overexpression in cancer cells promotes ferroptosis resistance.[8,9] Oxidative stress signals are closely related to

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cell proliferation and tumor growth. Due to the high metabolic rate of cancer cells, oxidative stress is mainly generated in cancer cells.[10,11] The activity of SLC7A11-mediated cystine uptake for synthesizing GSH in cancer cells is intricately linked to cell proliferation and tumor growth.[12]

Glutathione peroxidase 4 (GPX4) inhibits iron death by converting lipid hydroperoxides (L-OOH) to lipid alcohols (L-OH) and decreasing reactive oxygen species (ROS) levels.[13,14] Erastin or RSL3 can inactivate GPX4 and promote the process of lipid peroxidation, which ultimately leads to ferroptosis.[5,14] The increased expression of GPX4 in tumors is significantly correlated with tumor occurrence and metastasis. Inhibition or ablation of GPX enhanced lipid peroxidation chain reaction and induced ferroptosis in different cancer cells.[14] Yang et al demonstrated that ferroptosis inducer (RSL3) or inhibitor (liproxstatin-1) can inhibit the Nrf2/GPX4 signaling pathway to induce ferroptosis and enhance sensitivity to oxaliplatin in CRC.[15]

β-catenin is the primary mediators of the Wnt pathway. In the absence of WNT signaling, APC leads to degradation of β-catenin, preventing their accumulation in the cytoplasm by forming a complex with β-catenin, which leads to proteasomal phosphorylation and ultimately the destruction of β-catenin. WNT signaling blocks this process, allowing β-catenin to migrate from the cytoplasm to the nucleus. Once in the nucleus, β-catenin upregulates c-MYC, cyclin-D1, and other genes that increase cell proliferation.[16] Thus, continuous Wnt signaling is seen in APC-deficient cells.[17]

SLC7A11 and GPX4 have been well established as the negative regulator of ferroptosis, respectively.[18] Understanding the association of tumors with ferroptosis is crucial for several clinical and pathological characteristics. Although SLC7A11 and GPX4 were reported to be expressed in many types of cancers.[19–21] The objective of our study was to investigate the effect of SLC7A11 and GPX4 expression on clinical outcome and prognosis of colorectal cancer. Meanwhile, the expression of SLC7A11 and GPX4 in CRC and its relationship with Wnt/β-catenin have not yet been reported. We used immunohistochemistry to simultaneously detect the levels of SLC7A11, GPX4 and β-catenin, to explore the effects of SLC7A11, GPX4 on Wnt/β-catenin signaling pathway, their relationship, and prognosis.

In order to determine whether SLC7A11 and GPX4 can be used as markers for predicting the prognosis and survival of colorectal cancer, we used immunohistochemistry (IHC) to detect the expression of SLC7A11 and GPX4 in colorectal cancer tissues and studied the statistical relationship between the two. Then, we analyzed the relationship between the expression levels of SLC7A11 and GPX4 and the clinicopathological features (such as OS) of colorectal cancer patients.

Methods

Patients

This study selected 120 paraffin-embedded CRC samples diagnosed in First Affiliated Hospital of Bengbu Medical University from 2016 to 2017. Patients were evaluated in the clinical and histological study and for the survival analysis. A normal tissue sample 5 cm from the tumor margin was taken as a negative control. Of the 120 patients, 73 were male and ranged in age from 22 to 84 years, with a median age of 61 years. No patients had a history of chemotherapy or radiotherapy prior to surgery. The study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Bengbu Medical University (2023YJS131). This study was conducted in accordance with the Declaration of Helsinki. Each patient included in the study signed a written informed consent document. The clinical pathological parameters of the patients are presented in Table 1.

Histology

Fresh CRC specimens were taken after surgical resection, placed in 10% pH neutral formalin fixation, and embedded in paraffin. All patients were diagnosed with adenocarcinoma and staged, according to the American Joint Committee on Cancer (AJCC/TNM, 8th edition) staging system. Clinicopathological features included age, sex, tumor size, histological differentiation (good/moderate/poor, WHO), invasion depth, stage, and lymph node metastasis status. All tissue sections were reviewed by 2 senior pathologists at our institution to clarify the diagnosis and to assess the pattern and intensity of SLC7A11, GPX4 and β-catenin reactivity.

Table 1 Patients Characteristics

Patients Characteristics	Frequency (n)	Percentage (%)
Gender		
Man	73	60.8
Woman	47	39.2
Age (years)		
<60	49	40.8
≥60	71	51.2
Tumor sides (cm)		
<5.0	50	41.7
≥5.0	70	58.3
Grade		
Well	18	15.0
Moderate	81	67.5
Poor	21	17.5
LN metastasis		
Yes	49	40.8
No	71	59.2
TNM stage		
I+II	66	55.0
III+IV	54	45.0
Location		
colon	64	53.3
rectum	56	46.7

IHC

All tissue specimens were fixed in 10% formalin solution, embedded in conventional paraffin, and serially sectioned to a thickness of 4 μm, and baked at 60°C for 1 hr. All samples were dewaxed in xylene, dehydrated in alcohol, and washed with phosphate-buffered saline (pH 7.2) for 10 min. Endogenous peroxidase activity was blocked with 3% H2O2 in methanol, and slides were immersed in citrate buffer (pH 6.0) at 95°C for 30 min. The slides were incubated with goat serum for 30 min at room temperature and then incubated with SLC7A11 rabbit anti-human polyclonal antibody (1:200, 26,864-1-AP, Proteintech), GPX4 rabbit anti-human monoclonal antibody (1:500, AB125066, Abcam) and Monoclonal Mouse Anti-Human β-catenin (1:200, Dako) at 37°C for 1 h. Finally, freshly prepared diaminobenzidine solution was added to the slides, which were counterstained with hematoxylin, dehydrated, air-dried, and mounted.

Scoring Criteria

The immunohistochemical results of the sections were evaluated by two experienced pathologists. The immunohistochemical scores for SLC7A11 and GPX4 proteins were determined by the proportion of positive cells in tumor tissue (0, <10%; 1, 11–50%; 2, 51–75%; 3, >75%) and staining intensity (0, no staining; 1, pale yellow staining; 2, tan staining; 3, brown staining); the final score was the product of these two values. If the total score was ≥3, it was determined positive; otherwise, it was considered negative.

β-Catenin expression status was assessed based on the pattern of staining (cytoplasmic), intensity (0 to 4 +) and percentage of staining (0% to 100%). The staining would be considered negative if there was weak or no cytoplasmic expression, or positive if there was moderate or strong cytoplasmic expression.

Statistical Analyses

SPSS 26.0 was used for statistical analysis. Chi-square test was used to analyze the relationship between SLC7A11, GPX4 and β-catenin and clinicopathological parameters. Spearman correlation analysis was used to determine the correlation between SLC7A11, GPX4 and β-catenin. Kaplan–Meier analysis is used to compare OS factors. Univariate

and multivariate Cox regression models were used to determine the risk factors affecting prognosis. Statistical significance was set at $P < 0.05$.

Results

Relationships Between SLC7A11, GPX4, β-Catenin and Clinicopathological Parameters

We first analyzed the expression of SLC7A11, GPX4 and β-catenin in CRC using immunohistochemistry and statistical software. SLC7A11 is mainly expressed in cytoplasm and is significantly expressed in cancer tissues (60.8%, 73/120; Figure 1A and B) and rarely expressed in normal colon mucosal tissues (11.7%, 14/120; Figure 1C and D). The expression level of SLC7A11 in CRC tissues is related to lymph node metastasis (P=0.001) and TNM stage (P=0.001; Table 2) of the tumor and is independent of other parameters such as age and sex (P>0.05; Table 2). GPX4 is mainly located in the cytoplasm, and the expression rate of GPX4 in cancer tissues is (64.2%, 77/120; Figure 2A and B), which is higher than that in normal colon mucosa tissues (16.7%, 20/120; Figure 2C and D). GPX4 expression is related to lymph node metastasis (P=0.031) and TNM stage of tumor (P=0.015; Table 2) but is independent of other parameters (P>0.05; Table 2).

β-catenin was observed in 80 of 120 CRC tissue with cytoplasmic positivity (66.7%, 80/120; Figure 3). The expression of β-catenin was positively correlated with tumor grade (P=0.02), lymph node metastasis (P=0.001) and TNM stage (P=0.001; Table 2), but not with other parameters (P>0.05; Table 2).

Relationships Among SLC7A11, GPX4 and β-Catenin

Spearman correlation coefficient analysis showed that SLC7A11 expression was positively correlated with GPX4 expression (r=0.290, P<0.001) and β-catenin expression (r=0.410, P<0.001). Moreover, GPX4 expression was positively associated with β-catenin expression (r=0.209, P=0.022) (Table 3).

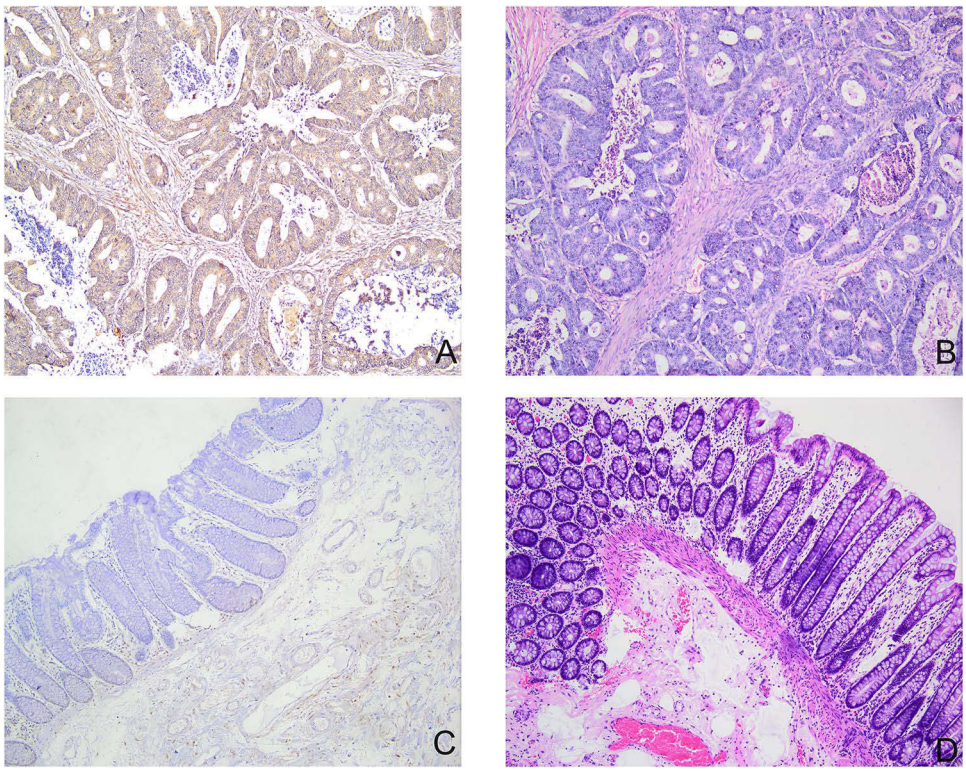

Figure 1 The expression of the SLC7A11 in CRC tissues and normal colon mucosal tissues (×100). (**A**) SLC7A11 staining in CRC tissues; (**B**) HE in staining in CRC tissues; (**C**) SLC7A11 staining in normal colon mucosal tissues; (**D**) HE in staining in normal colon mucosal tissues.

Table 2 Correlations Between SLC7A11, GPX4 and β-Catenin with Clinicopathological Characteristics of CRC

Variable	SLC7A11		P	GPX4		P	β-catenin		P
	Positive	Negative		Positive	Negative		Positive	Negative	
Sex			0.169			0.951			0.597
Male	48	25		47	26		50	23	
Female	25	22		30	17		30	17	
Age			0.225			0.546			0.599
<60	33	16		33	16		34	15	
≥60	40	31		44	27		46	25	
Tumor size			0.548			0.974			0.359
<5.0cm	32	18		32	18		31	19	
≥5.0cm	41	29		45	25		49	21	
Grade			0.218			0.262			**0.02**
Well	9	9		13	5		9	9	
Moderate	48	33		48	33		52	29	
Poor	16	5		16	5		19	2	
LN metastases			**0.001**			**0.031**			**0.001**
Yes	39	10		37	12		41	8	
No	34	37		40	31		39	32	
TNM			**0.001**			**0.015**			**0.001**
I+II	31	35		36	30		34	32	
III+IV	42	12		41	13		46	8	
Location			0.469			0.648			0.769
colon	37	27		40	24		42	22	
rectum	36	20		37	19		38	18	

Notes: Bold values indicate statistically significant.

Impact of SLC7A11 and GPX4 Expression on Prognosis

Univariate Prognostic Analyses

The total follow-up time was 70 months. Of the 120 patients, 50 patients were known to have died and 70 patients were still survived, the 5-years overall survival (OS) rate was 58.33%. Analysis of the effect of SLC7A11 combined negative or positive on OS (Figure 4). Patients with SLC7A11 high expression (39.422 ± 2.564 months) tended to have poorer prognosis than did patients with low expression (64.128 ± 2.255 months). The expression of SLC7A11 was negatively associated with the survival time of patients ($P < 0.01$; Log rank test). The survival time of patients with GPX4 positive (43.662 ± 2.735 months) was significantly shorter than that of patients with GPX4 negative (60.721 ± 2.767 months; $P < 0.05$; Log rank test) (Figure 5).

Multivariate Analysis and Cox's Proportional Hazard Model

According to the multivariate analysis, using all the influential factor variables SLC7A11 and GPX4. SLC7A11 expression (P=0.001) remained significantly associated with OS (HR = 4.733, 95% confidence interval, 1.964–11.408) and GPX4 expression (P=0.014) had no significant association with prognosis (HR = 2.572, 95% CI, 1.214–5.448). There was a trend toward poorer survival among the patients of SLC7A11 and GPX4 positive expression.

Discussion

The main findings of the present study are that SLC7A11 and GPX4 expression were significantly associated with advanced stage and implied poor prognosis in patients with CRC. In multivariate analysis, overexpression of SLC7A11 and GPX4 was an independent prognostic factor for OS. Meanwhile, in CRC tissue SLC7A11 and GPX4 may be related to the β-catenin.

As we all know, SLC7A11 and GPX4 are important regulators of ferroptosis.[5,18] SLC7A11, the catalytic subunit of a heterodimeric cystine/glutamate antiporter (System Xc–), transports extracellular cystine into cells for glutathione

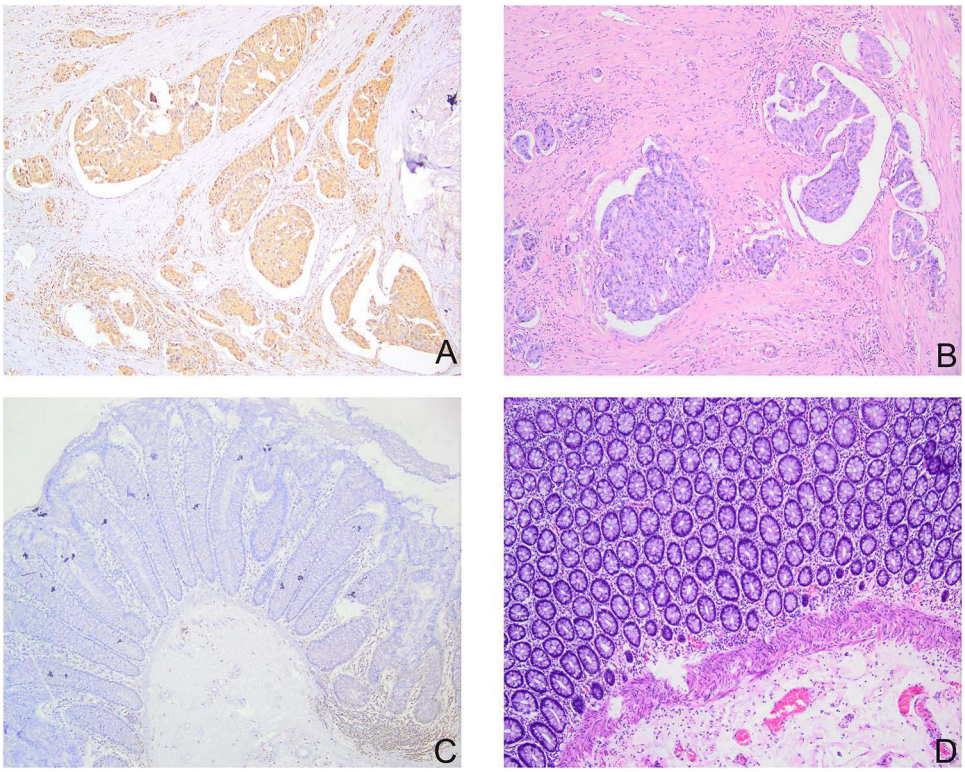

Figure 2 The expression of the GPX4 in CRC tissues and normal colon mucosal tissues (×100). (**A**) GPX4 staining in CRC tissues; (**B**) HE in staining in CRC tissues; (**C**) GPX4 staining in normal colon mucosal tissues; (**D**) HE in staining in normal colon mucosal tissues.

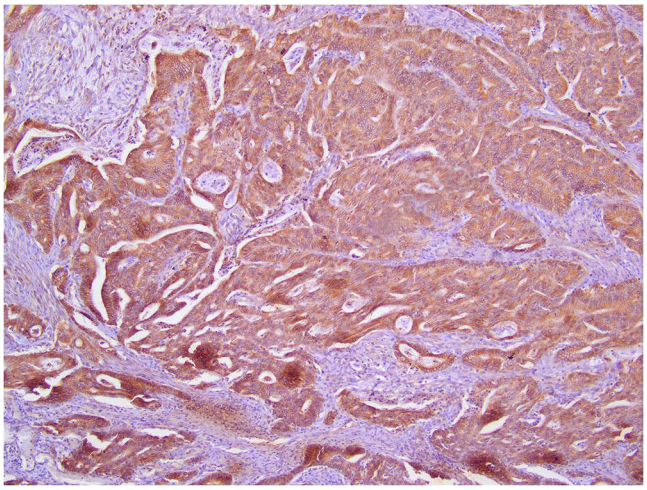

Figure 3 The cytoplasmic staining of β-catenin in CRC tissues (×100).

biosynthesis and is identified as a key regulator of ferroptosis. The knockdown or knockout of SLC7A11 significantly inhibited cancer cell growth, survival, and tumor formation by inducing ferroptosis.[22–24] The current study found that SLC7A11 expression was associated with sensitivity to iron death and that SLC7A11 conferred resistance to iron death in cancer cells. In KEAP1 mutant lung cancer tissues, where Nrf2 was constitutively activated, high expression of SLC7A11 significantly inhibited radiation-induced iron death, and the use of SLC7A11 inhibitors in an in vitro setting increased the sensitivity of cancer cells to radiotherapy.[25] Yang et al[26] found that in breast cancer metformin decreased the protein stability of SLC7A11 by inhibiting the process of ubiquitylation, which increased intracellular Fe2+ and lipid

Table 3 Correlation Among SLC7A11, GPX4 and β-Catenin in CRC

Variables	GPX4		*r*	*P*	SLC7A11		*r*	*P*
	Positive	Negative			Positive	Negative		
SLC7A11			0.290	<0.001				
Positive	55	18						
Negative	22	25						
β-catenin			0.209	0.022			0.410	<0.001
Positive	57	23			60	20		
Negative	20	20			13	27		

ROS levels, induced iron death and achieved tumor growth inhibition. In addition, SLC7A11 was also found to inhibit iron death in other malignant tumors such as gastric cancer, ovarian cancer and hepatocellular carcinoma.[27]

GPX4, one of the GPX subtypes, differs from other subtypes in that GPX4 can reduce lipid peroxides to lipid alcohols, thereby limiting the propagation of lipid peroxides in the cell membrane, and has the ability to degrade cell membrane lipid peroxides.[28] It has been shown that GPX4 plays an important role in tumor development, a variety of malignant tumors and were negatively correlated with patient survival. In hepatocellular carcinoma, the expression of GPX4 was significantly higher than that in normal hepatocellular tissues, and was significantly associated with the grade of hepatocellular carcinoma and clinical prognosis of patients.[29] In non-small cell lung cancer GPX4 has the property of maintaining tumor stem cells, while the resistance to radiotherapy may be related. In addition to malignant tumors such as breast cancer, nasopharyngeal carcinoma, gastric cancer, colorectal cancer, prostate cancer, diffuse large B-cell lymphoma and other malignant tumors GPX4 is highly expressed,[15,29] and Peng et al[30] found that GPX4 affects tumor epithelial mesenchymal transition (EMT) by regulating reactive oxygen species (ROS). Wang et al[21] show that in gastric cancer the beta-catenin/TCF4 transcription complex directly binds to the promoter region of GPX4 and induces its expression, resulting in the suppression of ferroptosis.

This study retrospectively assessed the immunohistochemical reactivity of SLC7A11 and GPX in CRC patients and investigated the relationship between their expression and clinicopathological features and prognosis. Our study shows that SLC7A11 was high expression in 60.83% (73/120) of CRC but low expression in normal mucosa. GPX4 was high expression in 64.17% (77/120) of tumors. The levels of expression of SLC7A11 and GPX4 in tumor cells were both higher than those in normal cells. This result is consistent with the main studies published to date.[31–34]

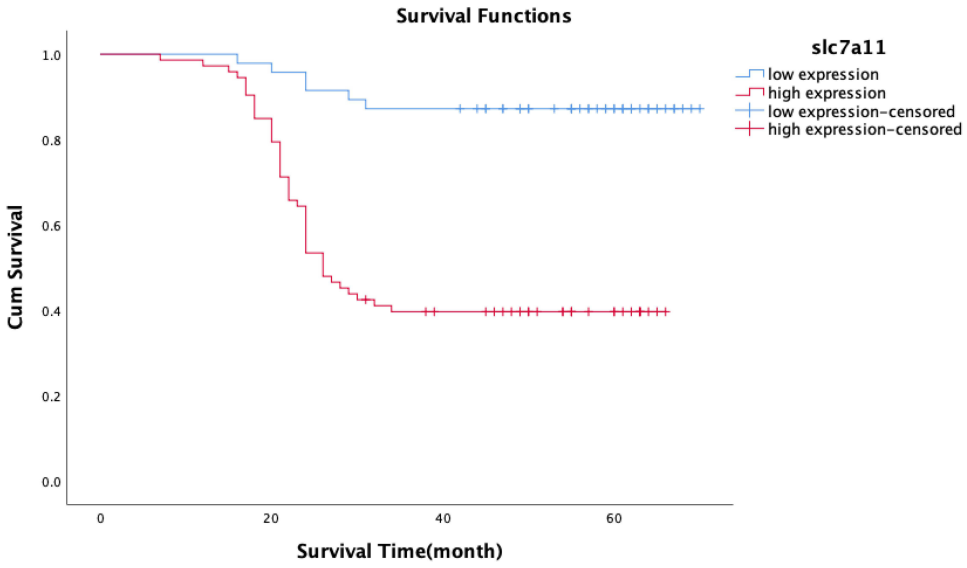

Figure 4 Impact of SLC7A11 expression on the patients' overall survival.

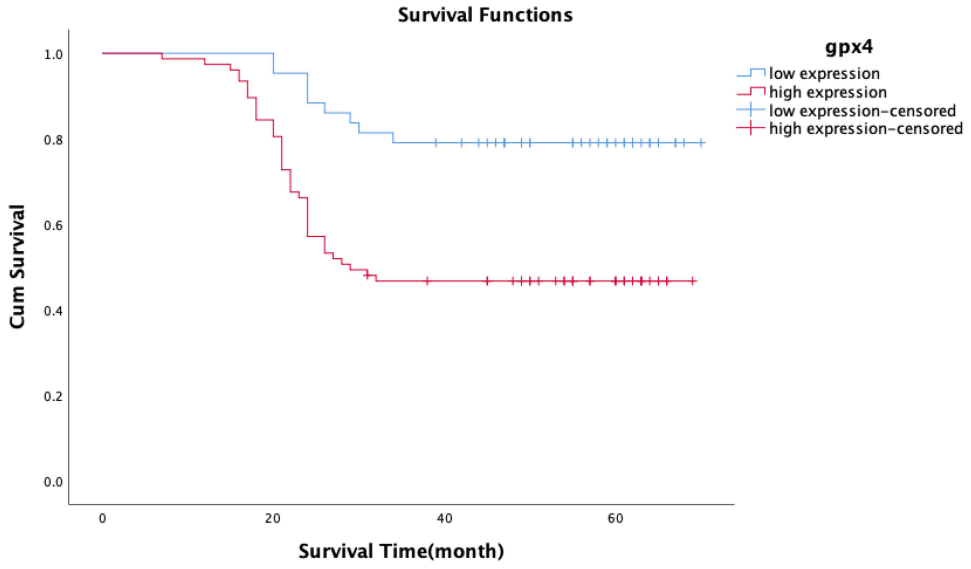

Figure 5 Impact of GPX4 expression on the patients' overall survival.

Through the research, we have found that the expression of SLC7A11 and GPX4 in different age, sex, tumor size, degree of histological differentiation, and invasion depth had no difference ($P > 0.05$). The difference of SLC7A11 and GPX4 expression in different stages of lymph nodes metastases had statistical significance ($P < 0.05$). The preliminary conclusion is that the high expression of SLC7A11 and GPX4 is clearly associated with advanced stage and appears to reflect more aggressive histological and clinical behavior. SLC7A11 and GPX4 can be used as biomarkers to indicate tumor invasion and metastasis assessment of CRC.

Previous studies have shown that positive SLC7A11 and GPX4 are associated with both TNM stage and lymph node metastasis of colorectal cancer.[35,36] In this study, we focused on the clinical and prognostic effects of SLC7A11 and GPX4 expression on patients with CRC. Our results showed that 5-year OS was 87.23% and 39.73% in patients with negative and positive expression SLC7A11, respectively. Patients with SLC7A11 high tumors had a significantly worse prognosis than those with SLC7A11 low tumors ($P<0.01$), with statistical significance. The 5-years OS of patients with low and high expression of GPX4 was 79.07% and 46.75%. The survival time of patients with GPX4 high expression was significantly shorter than that of patients with low expression ($P < 0.05$). As a result, the high expression of SLC7A11 and GPX4 both implied poor prognosis. Spearman analysis showed that SLC7A11, GPX4 and β-catenin were positively correlated in colorectal cancer. However, further studies are needed to determine the precise correlation between SLC7A11, GPX4 and β-catenin.

Based on the results of this study, we found that positive expression of SLC7A11 and GPX4 was associated with colorectal cancer invasiveness and patient survival time, patients with positive expression of SLC7A11 and GPX4 had a shorter survival time, a higher T-stage, as well as a greater tendency to develop lymph node metastasis. This result suggests whether SLC7A11 and GPX4 in colorectal cancer can be used as a new prognostic marker and risk group patients by positive expression level for better provision of therapeutic regimens, as in neuroendocrine tumors. These need to be studied with larger sample sizes and further experiments. Meanwhile, β-catenin is abnormally activated in colorectal cancer and is associated with related chemotherapy resistance, but the specific molecular mechanism is not clear.[37] The results of the present experiment showed that there was a positive correlation between SLC7A11 and GPX4 and β-catenin at the protein level, suggesting that there is a certain link between iron death and Wnt/β-catenin, which is more in line with related studies.[21] We speculate whether Ferroptosis can be regulated by modulating the Wnt/β-catenin signaling pathway to further improve the effectiveness of chemotherapy in patients with advanced colorectal cancer.

Conclusion

Ferroptosis plays an important role in tumor growth. SLC7A11 and GPX4 may be used as the biomarker to indicate tumor invasion and poor prognosis of CRC. However, the molecular mechanisms of SLC7A11 and GPX4 in colon cancer are still unclear and need to be further explored.

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Disclosure

The authors declare no competing interests.

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