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Cystathionine-β-synthase expression correlates with tumour progression and adverse prognosis in patients with colon cancer

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Abstract

Objective: To investigate the levels of cystathionine-β-synthase (CBS) in colon cancer tissues compared with adjacent control tissues; and to examine the relationship between CBS level and clinical characteristics and prognosis.

Methods: This retrospective study enrolled patients with primary colon cancer. Paraffin-embedded specimens were used to create pathological tissue microarrays. Immunohistochemistry was performed on the microarray to detect the levels of CBS in colon cancer tissues and normal adjacent tissues. Analyses were undertaken to examine the relationship between the level of CBS and clinical characteristics and prognosis.

Results: A total of 216 patients (107 males and 109 females) were included in the study. The level of CBS in cancer tissues was found to be significantly increased compared with normal adjacent control tissues. There were significant differences in tumour location, tumour-node-metastasis stage and survival rate between the CBS-negative and CBS-positive groups. Positive CBS immunostaining was associated with decreased survival in colon cancer patients. The results of multivariate Cox regression analysis revealed that tumour location and positive CBS immunostaining were independent prognostic factors for survival.

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Conclusion: Positive CBS immunostaining was closely associated with colon cancer and high levels of CBS might accelerate tumour development and affect patient prognosis in colon cancer.

Keywords
Cystathionine-β-synthase, colon cancer, immunohistochemistry

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Introduction

Colon cancer is the most common malignancy of the digestive tract in the world with increasing incidence and mortality in recent years.[1,2] Despite the remarkable success of multimodal cancer treatments, with surgery being the baseline modality,[3–5] the 5-year survival remains unsatisfactory.[6,7] There is a clinical need to identify sensitive tumour markers for colon cancer to allow prognosis and find new treatment targets.

The mechanism of action and expression pattern of endogenous hydrogen sulphide in cancer have attracted the attention of many scientists in the last two decades.[8–10] Cystathionine-β-synthase (CBS) is one of the key enzymes contributing to hydrogen sulphide (H_2S) biosynthesis and homocysteine metabolism.[8–12] CBS and H_2S synthesized by CBS both play various roles in regulating cellular energetics, redox status and DNA methylation.[13] Pathological upregulation of CBS and CBS-dependent H_2S production has been found in multiple cancer types, such as colon cancer,[11] ovarian cancer,[14] breast cancer,[15] prostate cancer[16] and renal cancer.[17] In 2013, a previous study reported for the first time the abnormal upregulation of CBS in human colon cancer tissues and colon cancer-derived cell lines and showed that inhibition of CBS expression and/or its activity has anti-tumour effects.[11] However, the detailed correlation between CBS expression and the clinical characteristics of colon cancer remains to be explored. Therefore, this current study aimed to further investigate the relationship between the level of CBS immunostaining and the clinical features and prognosis of patients with colon cancer.

Patients and methods

Patient population

This retrospective observational study enrolled consecutive patients with colon cancer in the Second Department of General Surgery, The Fourth Affiliated Hospital of Hebei Medical University, Shijiazhuang, Hebei Province, China between June 2013 and December 2014. The inclusion criteria were as follows: (i) patients with primary colon cancer; (ii) colon cancer pathologically confirmed by colonoscopy biopsy prior to surgery; (iii) no radiotherapy, chemotherapy or other clinical treatment prior to surgery; (iv) no previous history of tumours of other systems or a family history; (v) complete and clear clinical information; (vi) tumour surgery met the surgical standards for resection; (vii) the opinions of the patient and their family were sought. The exclusion criteria were as follows: (i) metastatic colon cancer; (ii) synchronous or heterochronous cancers; (iii) patients in whom the surgery was difficult to complete and those that were unwilling to accept surgical treatment; (iv) patients with anaemia and/or diabetes mellitus; (v) chronically undernourished patients with a body mass index $<17.0\,kg/m^2$.

All study participants provided signed informed consent. The study was approved by the Ethics Committee of the Fourth Affiliated Hospital of Hebei Medical University (no. ID2017MEC115; December 2017). The study conforms to STROBE guidelines and patient details have been de-identified.[18]

Clinical data collection

The following demographic and clinical data were collected from the patient records: sex, age, history of digestive diseases, smoking history, alcohol drinking history and postoperative pathology. The follow-up endpoint was patient death or the final follow-up on 1 January 2020.

Tissue sample preparation

Primary tumours with their normal adjacent tissues were obtained from surgical resections performed between June 2013 and December 2014 in the Second Department of General Surgery, The Fourth Affiliated Hospital of Hebei Medical University, Shijiazhuang, Hebei Province, China. For immunohistochemical analysis, formalin-fixed and paraffin-embedded (FFPE) tissues were collected and processed as described below.

CBS immunohistochemistry

Immunohistochemical experiments were performed using the streptavidin-peroxidase method.[19] Tumour and normal tissue samples were removed and 1 cm^3 of tissue was retained per sample, fixed in 10% neutral formalin, dehydrated with gradient ethanol and embedded in paraffin wax. These blocks were then used to prepare paraffin sections. The paraffin-embedded biopsy tissue blocks were cut into 4-μm sections. The tissue sections were placed in a rice cooker filled with citric acid antigen repair buffer (pH 6.0; Solarbio, Beijing, China) for antigen repair. The liquid in the rice cooker (5 l, 2000 W; SUPOR, Hangzhou, China) was boiled and the dewaxed sections were placed in the rice cooker for 15 min and excessive evaporation was prevented. After natural cooling, the sections were washed three times in 0.01 M phosphate buffered saline (PBS; pH 7.4; Solarbio) for 5 min each time. The sections were placed in a pressure cooker (3 l, 1500 W; SUPOR) containing EDTA antigen repair buffer (pH 8.0; Solarbio) for antigen repair for 3 min and excessive evaporation was prevented. After natural cooling, the sections were washed three times in PBS (pH 7.4; Solarbio) for 5 min each time. The tissue sections were then incubated overnight at 4°C in a moist chamber with the following primary antibody: mouse monoclonal antihuman CBS (Watson Biological Engineering, Hangzhou, China) at a 1:50 dilution. Then the sections were washed three times in PBS (pH 7.4; Solarbio) for 5 min each time. Protein staining was visualized using a labelled streptavidin-biotin-horseradish peroxidase kit (Hangzhou Watson Biological Engineering) according to the manufacturer's instructions. The CBS staining in the tissues was observed under a light microscope (DP26; Olympus, Tokyo, Japan). The results were independently assessed by three pathologists in a blinded manner. Inconsistency in the results were overruled by adopting the lower staining score in order to avoid subjective overestimation and false positives.

The proportion and immunostaining intensities of CBS-positive tumour cells were independently determined. The percentage of CBS-positive tumour cells was scored as follows: 0, no detectable staining; 1, 1–25% positive staining; 2, 26–50% positive staining; 3, 51–75% positive staining; 4, 76–100% positive staining. The intensity of staining was scored as follows: 0, no staining; 1, weak staining; 2, moderate staining; 3, high staining. The staining

index was the product of the intensity score multiplied by the percentage of stained cells. Thus, the minimum staining index score for CBS immunostaining was 0 and the maximum score was 12. A staining index score of <3 indicated negative CBS immunostaining and scores ≥ 3 indicated positive CBS immunostaining. Within the range of positive CBS immunostaining, scores of 3–4 indicated low immunostaining, 6–8 were moderate immunostaining and 9–12 were high immunostaining.

Statistical analyses

All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 26.0 (IBM Corp., Armonk, NY, USA). Data are summarized as frequencies (%) for and χ^2-test was used for between-group comparisons. The Kaplan–Meier method was used for estimating the survival curve and statistical comparisons between groups of different CBS levels were undertaken using the log-rank test. The Cox proportional hazards model was used to evaluate prognostic factors. A *P*-value <0.05 was considered to be statistically significant.

Results

This retrospective observational study enrolled 216 patients with primary colon cancer; including 107 men and 109 women. Their mean age was 59.6 years. Of the 216 patients, 109 patients had left-sided colon cancer and 107 had right-sided colon cancer. The follow-up endpoint was patient death or the final follow-up on 1 January 2020. The median follow-up was on 1 June 2019.

A total of 216 pathological tissue sections were stained with anti-CBS antibody. CBS was found to be present in both the cytoplasm and nucleus of the cells. The staining appeared as brown-yellow granules (Figure 1). The results demonstrated that 140 of 216 (64.8%) patients had positive CBS immunostaining in the colon cancer tissues, while only 11 of 216 (5.1%) patients had positive CBS immunostaining in the normal adjacent mucosal tissues. There were significantly higher levels of CBS immunostaining in the colon cancer tissues compared with the normal adjacent tissues (χ^2-test $= 118.021$; $P < 0.001$).

The patients were stratified according to the levels of CBS immunostaining (negative versus positive) of the colon cancer tissues (Table 1). Positive CBS immunostaining was significantly correlated with tumour location, tumour-node-metastasis (TNM) stage and survival rates ($P < 0.05$ for all comparisons). There were no significant differences observed with regard to sex, age, prior medical history, family history, smoking history, alcohol drinking history, pathological results, degree of differentiation, lymph node metastasis, T staging and N staging.

Univariate Cox regression analysis to evaluate the demographic and clinical characteristics affecting patient survival demonstrated that tumour location, immunohistochemistry results, prior medical history and TNM stage were significant prognostic factors ($P < 0.05$), while other characteristics did not affect patient prognosis. Multivariate Cox regression analysis demonstrated that tumour location and immunohistochemistry results were significant prognostic factors for patient survival ($P < 0.05$), while other characteristics did not affect patient prognosis (Table 2). The survival rate of patients with right-sided colon cancer was lower than that of patients with left-sided colon cancer. CBS-positive patients had a reduced survival rate compared with CBS-negative patients. Among the 216 patients followed up for 6 years and 4 months, the survival rate of CBS-negative patients was 69.1% and CBS-positive patients was 45.4%. The median survival time was 67 months. The 5-year survival rates were 77.0% for CBS-negative patients

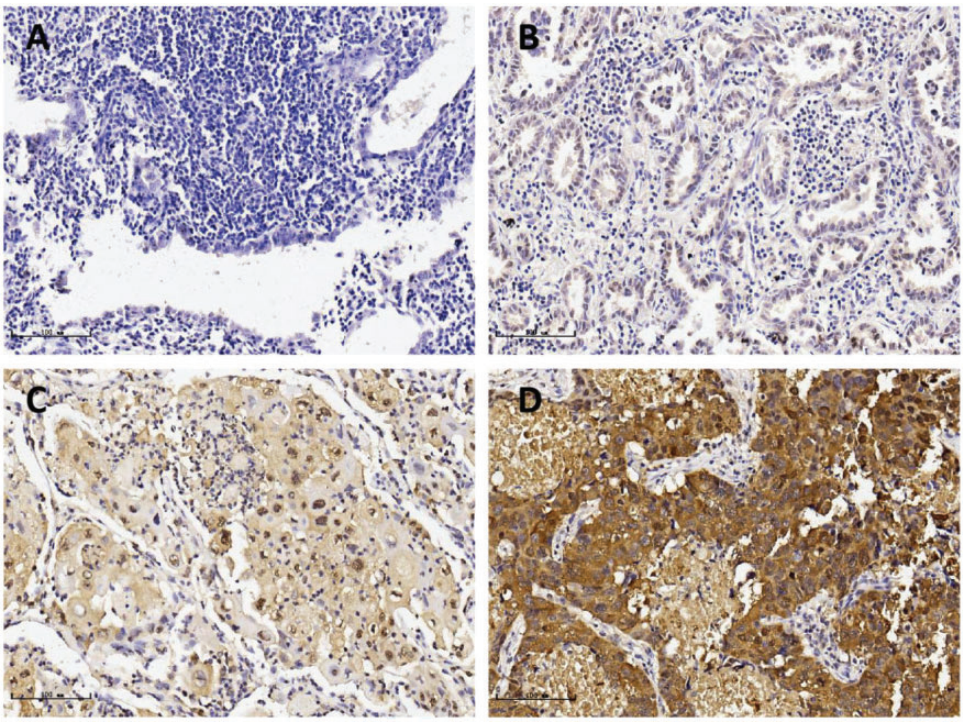

Figure 1. Representative photomicrographs of immunohistochemical staining of colon cancer tissues for cystathionine-β-synthase (CBS): (a) CBS negative levels of staining of colon cancer tissues; CBS positive levels of staining of colon cancer tissues ((b) low immunostaining; (c) moderate immunostaining and (d) high immunostaining). Scale bar, 100 μm. The colour version of this figure is available at: http://imr.sagepub.com.

and 55.0% for CBS-positive patients. Survival curves were estimated using the Kaplan–Meier method and intergroup comparisons were undertaken using the log-rank test. The results showed a significant difference in survival between the CBS-negative and CBS-positive groups ($\chi^2 = 8.904$; hazard ratio 2.032; 95% confidence interval 1.261, 3.274; $P = 0.003$) with a higher survival in CBS-negative group (Figure 2).

Discussion

Colon cancer is a global socioeconomic crisis. Although biomarker testing (such as microsatellite instability, K-Ras, BRAF, HER-2) has laid a foundation for the precision treatment for colon cancer,[20] there remains a dire need to search for novel therapeutic approaches targeting tumour-specific pathways. Multiple studies have shown that CBS levels are closely associated with tumour metabolism and prognosis and CBS may act as a potential biomarker.[14–17]

Cystathionine-β-synthase is primarily a cytoplasmic protein, but it has also been found in various other cellular components. For example, CBS has been shown to enter the nucleus;[21] and in hypoxic or ischaemic conditions, CBS can be translocated to the mitochondria.[22] In cancer cells, cytoplasmic and mitochondrial CBS were reported in a preliminary study in 2013.[14] Since mitochondrial H_2S can stimulate a variety of processes supporting the cellular energy and vitality of cancer cells,[23–26] mitochondrial translocation of CBS may play an

Table 1. Demographic and clinical characteristics of patients ($n = 216$) with colon cancer stratified according to the levels of cystathionine-β-synthase (CBS) immunohistochemical staining (negative versus positive) of the colon cancer tissues.

Characteristic	*n*	Immunohistochemical results		χ^2-test	Statistical analysis[a]
		CBS negative $n = 76$	CBS positive $n = 140$		
Sex				0.034	NS
Male	107	37	70		
Female	109	39	70		
Age, years				0.062	NS
<60	97	35	62		
≥60	119	41	78		
Tumour location				4.750	$P = 0.029$
Left hemicolon	109	46	63		
Right hemicolon	107	30	77		
Prior medical history				0.777	NS
No	175	64	111		
Yes	41	12	29		
Family history				0.029	NS
No	172	61	111		
Yes	44	15	29		
Smoking history				2.311	NS
No	161	52	109		
Yes	55	24	31		
Alcohol drinking history				0.000	NS
No	159	56	103		
Yes	57	20	37		
Pathological result				0.399	NS
Adenocarcinoma	170	58	112		
Other	46	18	28		
Differentiation degree				0.923	NS
Low	6	1	5		
Moderate	210	75	135		
T stage				4.546	NS
T1	3	1	2		
T2	15	8	7		
T3	52	22	30		
T4	146	45	101		
N stage				0.214	NS
N0	130	46	84		
N1	71	24	47		
N2	15	6	9		
TNM stage				4.889	$P = 0.027$
1–2	129	53	76		
3–4	87	23	64		
Lymph node metastasis				0.017	NS
No	132	46	86		
Yes	84	30	54		

(continued)

Table 1. Continued.

Characteristic	*n*	Immunohistochemical results		χ^2-test	Statistical analysis[a]
		CBS negative $n=76$	CBS positive $n=140$		
Survival status					
Survival	121	54	67	10.757	$P=0.001$
Death	95	22	73		

Data are presented as *n* of patients.
[a]χ^2-test was used for between-group comparisons; NS, no significant between-group difference ($P \geq 0.05$).
T, tumour; N, node; TNM, tumour-node-metastasis.

Table 2. Multivariate Cox regression analysis of potential prognostic demographic and clinical characteristics for survival in patients ($n=216$) with colon cancer.

Characteristic	*n*	5-year survival rate, %[a]	HR	95% CI	Wald	Statistical analysis
Sex			1.110	0.722, 1.708	0.228	NS
Male	107	59.8				
Female	109	56.4				
Age, years			0.719	0.463, 1.118	2.141	NS
<60	97	54.3				
≥60	119	60.1				
Tumour location			1.723	1.047, 2.836	4.580	$P=0.032$
Left hemicolon	109	66.0				
Right hemicolon	107	48.7				
Immunohistochemical results for CBS			1.735	1.028, 1.929	4.256	$P=0.039$
Negative	76	77.0				
Positive	140	55.0				
Prior medical history			1.450	0.864, 2.434	0.979	NS
No	175	68.3				
Yes	41	39.6				
Family history			1.176	0.696, 1.987	0.367	NS
No	172	67.2				
Yes	44	49.9				
Smoking history			1.625	0.850, 3.107	2.153	NS
No	161	64.8				
Yes	55	55.3				
Alcohol drinking history			0.615	0.321, 1.180	2.136	NS
No	159	64.5				
Yes	57	57.7				
Pathological result			0.623	0.343, 1.132	2.415	NS
Adenocarcinoma	170	65.7				
Other	46	61.3				
Differentiation degree			1.127	0.282, 4.500	0.028	NS
Low	6	50.0				
Moderate	210	72.0				

(continued)

Table 2. Continued.

Characteristic	*n*	5-year survival rate, %[a]	HR	95% CI	Wald	Statistical analysis
T stage						
T1	3	66.7	1.000			
T2	15	80.0	0.606	0.064, 5.705	0.192	NS
T3	52	62.8	1.560	0.207, 11.766	0.186	NS
T4	146	59.8	1.208	0.158, 9.209	0.033	NS
N stage						
N0	130	67.5	1.000			
N1	71	54.4	1.504	0.172, 13.171	0.136	NS
N2	15	57.4	1.387	0.125, 15.423	0.071	NS
TNM stage			0.702	0.341, 1.446	0.920	NS
1–2	129	70.2				
3–4	87	51.2				
Lymph node metastasis			0.989	0.122, 8.030	0.000	NS
No	132	67.1				
Yes	84	50.7				

Data are presented as *n* of patients and percentage survival.

[a]Percentage survival data were calculated using survival analysis software and were not simply the percentage obtained by dividing the numerator by the denominator.

NS, no significant between-group difference ($P \geq 0.05$).

HR, hazard ratio; CI, confidence interval; CBS, cystathionine-β-synthase; T, tumour; N, node; TNM, tumour-node-metastasis.

important role in regulating cancer cell energetics. In addition, CBS also regulates NF-κB and p53-related apoptotic pathways.[14] A recent study indicated that CBS is involved in nucleolar stress-induced apoptosis.[27] It has also been shown that CBS is upregulated in tumour tissues in colon, ovarian and prostate cancers, generating endogenous H_2S, regulating vascular endothelial growth factor (VEGF) expression in endothelial cells and promoting tumour angiogenesis.[28–31] A previous study demonstrated that the CBS-H_2S axis promotes liver metastasis of colon cancer by upregulating VEGF expression.[32] This current study is one of the first to clinically validate the previously reported results with a larger sample size.

This current study demonstrated that CBS is overexpressed in colon cancer tissues and its level increases with tumour staging. Patients with positive CBS immunostaining had a lower survival rate than CBS-negative patients, suggesting that endogenous H_2S produced by CBS may be involved in the proliferation or metastasis of colon cancer. This finding also indicated that CBS may play an important role in the development and prognosis of colon cancer. In addition, this current study found that a significantly higher proportion of patients with right-sided colon cancer (77 of 107 patients; 72.0%) had CBS positive tumours compared with those with left-sided colon (63 of 109 patients; 57.8%) ($P = 0.029$); and the survival rate was significantly lower in patients with right-sided colon cancer ($P = 0.032$). This result was consistent with the clinical observation that survival of right-sided colon cancer patients is poorer than those of left-sided colon.[33] However, not all adjacent groups showed statistically

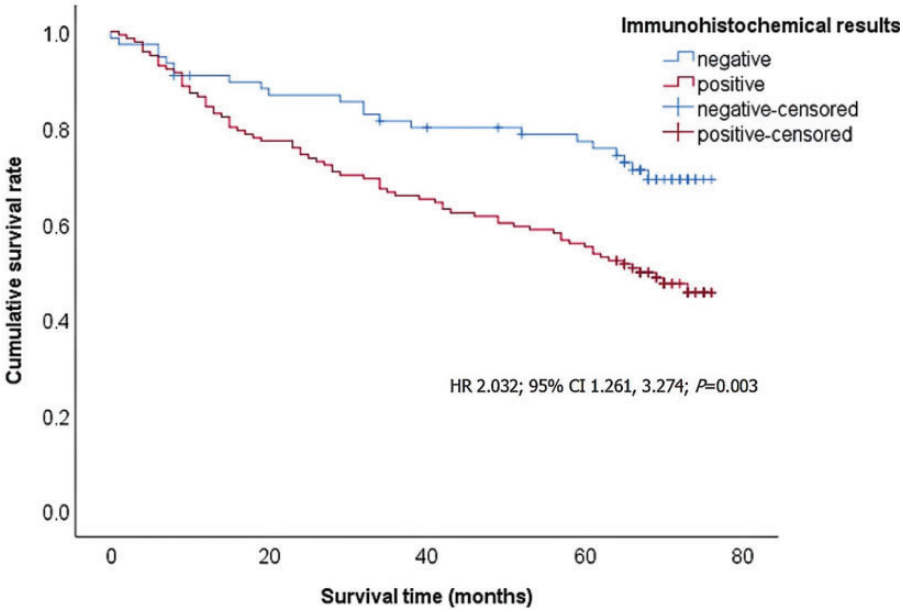

Figure 2. Kaplan–Meier survival curves of patients ($n = 216$) with colon cancer stratified according to the levels of cystathionine-β-synthase immunohistochemical staining (negative versus positive) of the colon cancer tissues. HR, hazard ratio; CI, confidence interval. The colour version of this figure is available at: http://imr.sagepub.com.

significant differences, indicating that the expression of CBS in tumour development may be gradual and complex.

This current study, for the first time, explored the relationship between CBS immunostaining and the clinical features of colon cancer in a large patient group. In future, combined with other molecular markers and clinical features, predictive models can be established to achieve individualized treatment strategies. However, this current study had some limitations. First, it was a retrospective data analysis, with potential biases and limitations. Secondly, the sample size was relatively small compared with the overall population, so further larger scale studies are needed to verify the reliability of the current results. Thirdly, the specific mechanism of action of CBS in colon cancer was not investigated extensively so further experiments are needed to explain its function and regulatory mechanisms. The results of this current study provide the basis for further *in vivo* and *in vitro* experiments to explore the potential mechanisms of action of CBS in colon cancer. Multicentre clinical studies are needed to verify the reliability of CBS as a prognostic marker for colon cancer.

In conclusion, this current retrospective observational study demonstrated that CBS is highly expressed in colon cancer tissues and is associated with its prognosis. High levels of CBS might influence tumour progression and be associated with poor prognosis in colon cancer. In the postoperative assessment of colon cancer patients, CBS might serve as a potential biomarker for predicting patient prognosis.

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Author contributions

Xiao-Jie Hu and Yun Sun wrote the manuscript; Guang-Jie Liu and Juan Zhang contributed to the data collection; Yang-Hui Peng and Li-Xiao Zhang contributed to the manuscript discussion.

Availability of data and materials

The datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request.

Declaration of conflicting interest

The authors declare that there are no conflicts of interest.

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