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Original Article

Cystathionine β-synthase expression correlates with tumor development and poor prognosis in patients with adenocarcinoma of the gastroesophageal junction

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Abstract: Objectives: To reveal the expression level of cystathionine β-synthase (CBS) in adenocarcinoma of esophagogastric junction (AEG) and discuss the relationship between CBS expression level and tumor microvascular density (MVD), clinical features and prognosis. Methods: Paraffin samples from 214 patients with AEG were selected to make pathological microchips. Immunohistochemistry was performed based on the microchips to detect the expression level of CBS and microvascular density (MVD) in cancer tissues and adjacent control tissues. Relationships between expression level of CBS and MVD, clinical characteristics and prognosis were analyzed. Results: In total, 214 AEG cases were classified into three groups: CBS negative staining (n=26), low staining (n=44), and high staining (n=144). Quantitative alterations in CBS and CD31 expression were explored using immunohistochemistry. The 5-year recurrence rate of enrolled patients was followed up and found that CBS expression was significantly increased in tumor tissue compared with adjacent non-tumor tissue (P<0.0001). There were significant differences in microvascular density between the groups with negative and high CBS staining (P<0.0001), and between the groups with low and high CBS staining (P<0.0001). Univariate analysis revealed significant differences in tumor stage (P<0.0001), T stage (P=0.008), N stage (P=0.028), differentiation degree (P=0.037), and 5-year survival (P=0.0034) among the three groups. Multivariate logic regression analysis showed that increased CBS scores were associated with an increased probability of 5-year recurrence (P=0.018). Finally, different CBS expression levels were associated with disease-free survival in AEG patients. Conclusions: CBS expression level is closely related to microvascular density and tumor stage in AEG. High level of CBS not only accelerates tumor angiogenesis but also affects patient's survival and prognosis.

Keywords: Cystathionine β-synthase, adenocarcinoma of the gastroesophageal junction, immunohistochemistry

Introduction

An ongoing increase in the incidence of adenocarcinoma at the gastroesophageal junction (AEG) has occurred in recent decades [1]. Surgical treatment is the primary therapeutic option for AEG, which is considered a unique clinical malignancy with different clinicopathological features, etiology, and biological behaviors than esophageal squamous cell carcinoma and gastric carcinoma [2]. AEG is defined as a malignancy that transverses the esophagogastric junction, including distal esophageal adenocarcinoma and proximal gastric cancer [3, 4]. Chronic gastroesophageal reflux disease is a strong risk factor for AEG that leads to intestinal metaplasia [5]. Interestingly, *Helicobacter pylori* infection, which is a risk factor for gastric cancer, is considered a protective factor for AEG, as it can prevent reflux esophagitis and Barrett's esophagus [6]. The treatment strategy for AEG is complex because of the anatomical location of the esophagogastric junction. The incidence of AEG has shown an increasing trend over the past few decades, and AEG patients have a poor prognosis [7, 8]. AEG patients have poorer outcomes than esophageal cancer and gastric adenocarcino-

ma patients, and the long-term survival rate of AEG remains unsatisfactory, despite new treatments being tested in the clinic. Therefore, there is an urgent need to identify sensitive tumor markers for AEG that will allow prognostic evaluations and the clinical monitoring of AEG patients for recurrence [9, 10].

Cystathionine β-synthase (CBS) regulates homocysteine (Hcy) metabolism and contributes to hydrogen sulfide (H_2S) biosynthesis. Through these activities, CBS plays multifunctional roles in the regulation of cellular energetics, redox status, DNA methylation, and protein modifications [11]. Increased CBS expression has been found in multiple tumor types, such as colon cancer, ovarian cancer, bladder cancer, breast cancer, kidney cancer, and oral squamous cell carcinoma [12-17]. In some tumors, high CBS expression usually predicts poor clinical prognoses [12, 14, 16, 17]. CBS regulates tumor growth and survival at multiple levels and can promote tumor cell survival by increasing the cell intrinsic antioxidant capacity. Ovarian cancer cells depleted of CBS showed enhanced production of reactive oxygen species [14, 18]. Additionally, CBS regulates the NF-κB and p53 apoptosis-related pathways [14]. A recent study further suggested that CBS is involved in nucleolar stress-induced apoptosis [19].

Thus, CBS is highly expressed in multiple tumor types, where it is involved in pathways related to tumorigenesis and development. Therefore, we hypothesized that CBS was also highly expressed in AEG and that different CBS levels could directly affect the survival and prognosis of AEG patients. Tumor growth is closely related to angiogenesis; therefore, we further explored correlations between CBS expression and tumor angiogenesis. Finally, this study proved that CBS was a risk factor that affected the prognosis of AEG patients.

Methods

Patients and sample preparation

This study was approved by the Institutional Review Committee of the Fourth Hospital of Hebei Medical University (ID2018MEC108). All patients signed informed consent form for the use of their tissue in this study. Primary tumors with their adjacent tissues were obtained from surgical resections that occurred between January 2013 and June 2014. Formalin fixed and paraffin embedded (FFPE) tissues were selected for analysis. Surgical records and preoperative radiological images were used to re-stage the tumors according to the 2020 National Comprehensive Cancer Network AEG Treatment Guidelines [20]. Diagnoses of tumors and adjacent tissues were confirmed from hematoxylin and eosin (H&E)-stained slides by a pathologist at the Fourth Hospital of Hebei Medical University. In total, 214 patients who underwent resection of primary AEGs were enrolled. Clinical outcomes including disease-free survival (DFS) were determined by chart review. The follow-up endpoint was postoperative relapse, and the final follow-up date was November 31, 2020. Histology, clinical staging, patient demographics, and analyzed lesions are summarized in **Table 1**.

CBS immunohistochemistry (IHC)

Representative tumor regions and paired normal tissue regions were selected from H&E-stained pathological sections and labeled. Then, samples with diameters of 600 μm were collected from the corresponding region of the FFPE block specimen according to the labeled regions in the sections. FFPE samples were then placed into the paraffin block bracket of the micromatrix chip to prepare the pathological chips. Finally, 4-μm thick sections were used for subsequent examinations.

The microarray pathological chips were stained using a Ventana Discovery XT automated system (Ventana Medical Systems, Tucson, AZ, USA) and proprietary reagents according to the manufacturer's protocol. Briefly, slides were deparaffinized using the automated system with EZ Prep solution (Ventana Medical Systems). The heat-induced antigen retrieval method was used in Cell Conditioning 1 solution (Ventana Medical Systems). A mouse monoclonal antibody to human CD31 (Abcam, Cambridge, UK) and a mouse monoclonal antibody to human CBS (Abcam) were used at a 1:1,000 dilution in Dako antibody diluent (Dako, Carpentaria, CA, USA) and incubated for 60 min. Ventana anti-mouse secondary antibodies were then incubated with the sections for 16 min. The detection system used was The Ventana OmniMap kit. Slides were then dehydrated and cover-slipped, according to standard laboratory protocols.

Evaluation of CBS staining

Relative CBS protein expression was determined from immunostaining intensities, which

Table 1. Demographics and baseline characteristics of patients with different CBS expression levels (n=214)

Variable		No. of cases (n, %)	CBS Negative expression	CBS Low expression	CBS High expression	*P* value
			n=26	n=44	n=144	
Age (median, range)			60 (51-71)	61 (51-79)	61 (54-79)	0.679
Sex						0.270
	Male	78 (36%)	12 (46%)	19 (43%)	59 (41%)	
	Female	136 (64%)	14 (54%)	25 (75%)	85 (59%)	
Differentiation						0.037
	Highly differentiated	32 (15%)	6 (23%)	8 (18%)	18 (12%)	
	Moderately differentiated	117 (55%)	18 (69%)	18 (41%)	81 (56%)	
	Poorly differentiated	65 (30%)	2 (8%)	18 (41%)	45 (31%)	
HER-2 expression	Positive expression	16 (7%)	3 (12%)	5 (11%)	8 (6%)	0.059
T stage	T stage I-II	63 (29%)	10 (%)	13 (%)	40 (28%)	0.008
	T stage III-IV	151 (71%)	16 (%)	31 (%)	104 (72%)	
N stage	N stage 0 (Negative)	196 (92%)	22 (%)	32 (%)	81 (56%)	0.028
	N stage 1, 2, 3 (Positive)	18 (8%)	4 (%)	12 (%)	64 (44%)	
TNM stage						<0.0001
	Stage I-II	179 (84%)	24 (92%)	42 (95%)	131 (91%)	
	Stage III	35 (16%)	2 (8%)	2 (5%)	34 (24%)	
Smoking history		80 (37%)	10 (38%)	14 (32%)	56 (39%)	0.113
Drinking history		174 (81%)	16 (62%)	30 (68%)	128 (89%)	0.062
No. recurrences within 5 years		91 (43%)	10 (38%)	59 (62%)	98 (68%)	0.0034

were scored on a 0 to 3 scale as follows: no staining, 0; weak staining, 1; moderate staining, 2; and high staining, 3. The percentage of cells stained was measured as follows: no detectable staining, 0; 1-25% positive staining, 1; 26-50% positive staining as 2, 61-75% as 3, and finally 76-100% as 4. The final IHC score was the product of the percentage of stained cells multiplied by the intensity score, allowing for a minimum score of 0 and a maximum score of 12. We defined the final score of 0 as negative CBS expression (0 points), 1-2 as low CBS expression (1 point), and product of the percentage of stained cells multiplied by the intensity score, allowing for a minimum score of 0 and a maximum score of 12. We defined the final score of 0 as negative CBS expression (0 points), and 1 as focleus and cytoplasm were measured and quantified. At the same time, we used CD31 to mark microvessels and calculate the microvascular density (MVD) as the number of microvessels per mm^2.

Statistical analysis

Nonparametric statistical tests were used for data analysis. Data are summarized as median (range) for continuous variables, and frequencies (%) for categorical variables. We applied the chi-square test to calculate the difference between nominal variables under situations with different CBS expression. The Wilcoxon signed-rank test was used to calculate the difference in patient ages in the groups of different CBS expression levels. The correlation between CD31 and CBS was analyzed by the Kendall coefficient. A multivariable logistic regression analysis was used to determine potential variables that could predict the risk of CBS. All statistical analyses were performed using SPSS statistical package (version 21.0; IBM Corp., Armonk, NY, USA). Figures were generated using GraphPad Prism (version 7.0; GraphPad Software, Inc., San Diego, CA, USA).

Results

Clinical characteristics of AEG patients

Patients' demographics, clinical characteristics, and tumor statuses are presented in **Table 1**. This study enrolled 214 patients, including 78 men (36%) and 136 women (64%). Pathological sections from the patients were classified into three groups according to the different degrees of differentiation, including the low, moderate, and high differentiation groups.

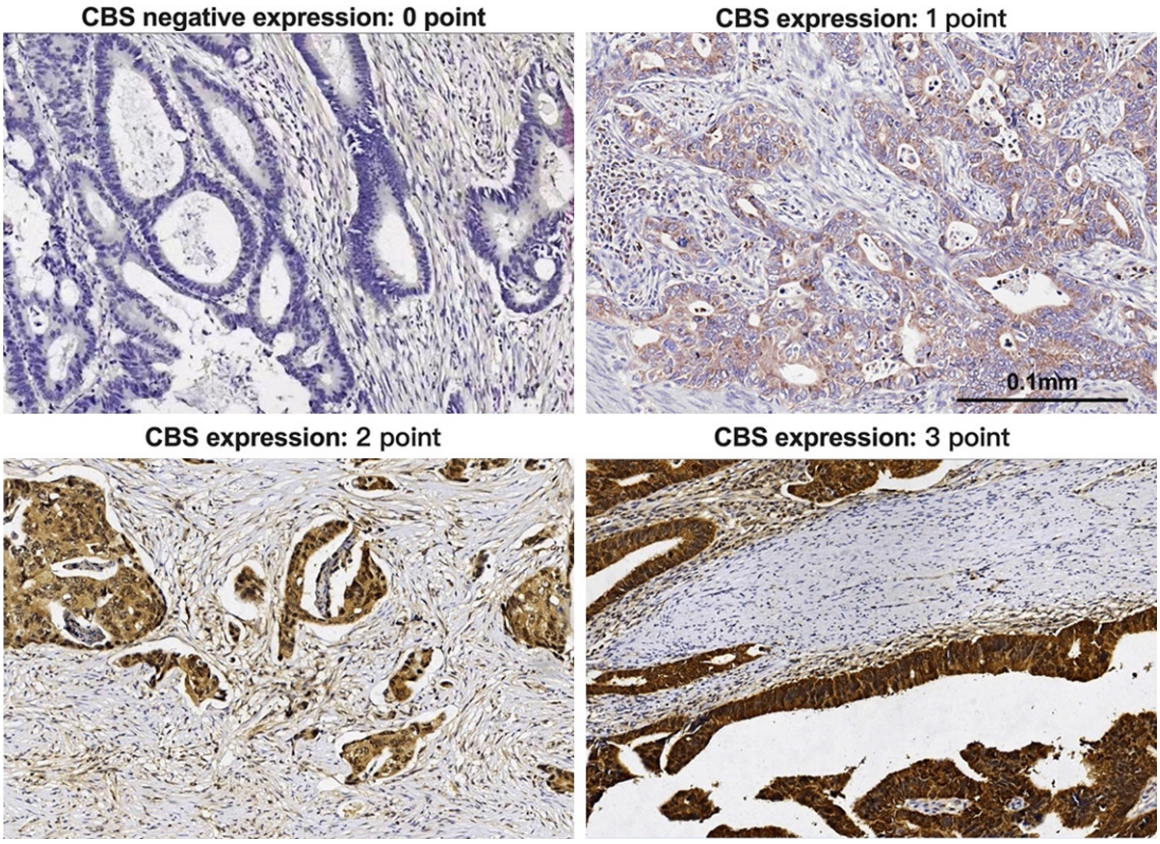

Figure 1. CBS expression in tumor tissues from AEG patients.

Among them, the greatest number of patients (*n*=117, 55%) were in the moderate differentiation group, whereas the least number of patients (*n*=32, 15%) were in the high differentiation group. According to the eighth edition of the International Union against Cancer TNM classification, this study enrolled resectable patients with stage I-III disease. Among these cases, 179 (84%) had early-stage (stage I-II) disease and 35 (16%) had locally advanced (stage III) disease. The distribution of tumor (T) staging among the cohort included 18 cases (8.4%) of T1, 45 cases (21.0%) of T2, 89 cases (41.6%) of T3, and 62 cases (29%) of T4 disease. There were 135 (63.1%) node (N) negative patients and 79 (36.9%) N positive patients. Additionally, 37% of patients had a smoking history and 81% of patients had a drinking history. Among the CBS negative expression group, 73% (19/26) of patients accepted platinum-based chemotherapy (capecitabine, 2500 mg/m^2 and oxaliplatin, 130 mg/m^2 q3W) after surgery, while in the low and high expression groups, 89% (39/44) and 89% (128/144) of patients accepted the treatment after surgery; there were no statistically significant differences in the therapy among the three groups ($P>0.05$). The 5-year recurrence rate of the enrolled patients after surgery was followed up, and 91 patients (43%) did not suffer from recurrence at 5 years after surgery.

We stained 214 pathological tissue sections with CBS antibody, and then divided them into three groups according to different CBS expression levels. We also performed univariate analysis of various clinical characteristics of the patients, which revealed no statistically significant differences in age, sex, smoking history, post-operative therapy, or drinking history between the groups. However, differences in differentiation degree ($P=0.037$), TNM classification ($P<0.0001$), and 5-year tumor recurrence ($P=0.006$) were statistically significant among the groups divided by CBS expression level (**Table 1**).

CBS expression was significantly increased in tumor tissue compared with adjacent non-tumor tissue and increased with advanced stage

Tumor and adjacent non-tumor tissue from the 214 patients were stained with CBS (**Figure 1**). The results demonstrated that 67.29% of

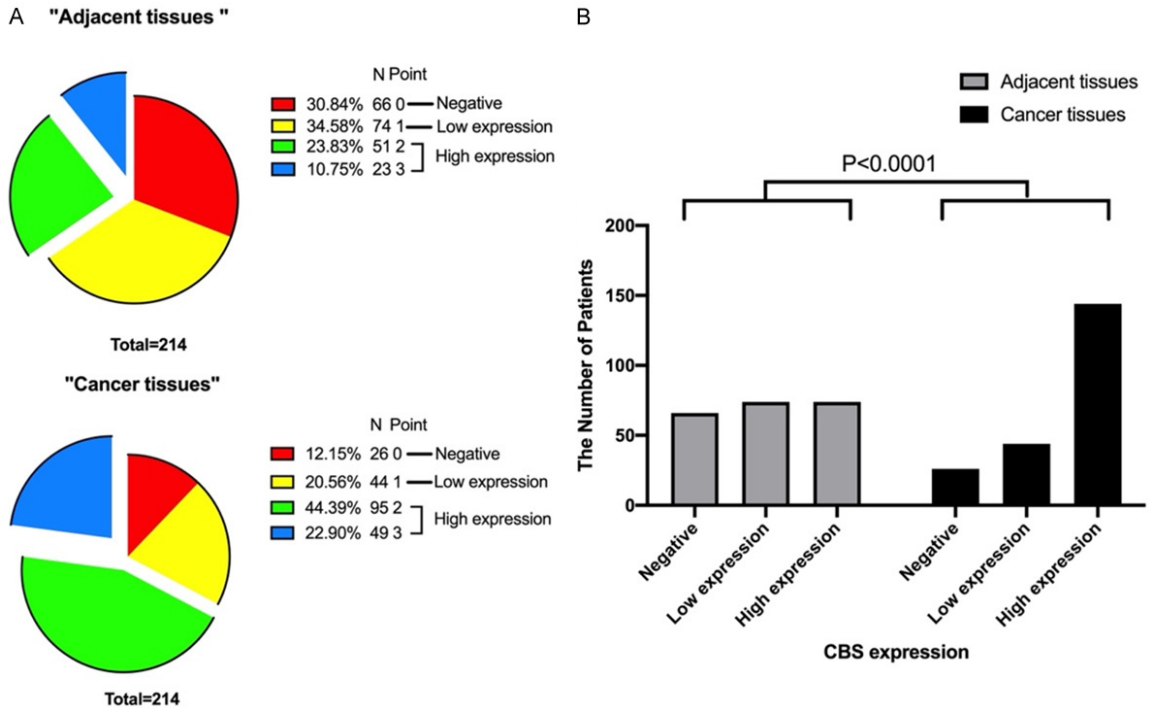

Figure 2. CBS expression was significantly increased in tumor tissues compared with adjacent no-tumor tissues. A: In total, 66% of AEG patients had high CBS expression in tumor tissues. Regarding adjacent no-tumor tissues, 33% had high CBS expression. B: CBS showed high expression in tumor tissues (P<0.0001).

patients had high CBS expression in tumor tissue, while only 34.58% of patients had high CBS expression in adjacent non-tumor tissue. In contrast, 20.56% of patients had low CBS expression in tumor tissue, while 34.58% had low CBS expression in adjacent non-tumor tissue (**Figure 2A**). Then, the chi-square test was used to evaluate the difference in CBS expression between adjacent non-tumor and tumor tissues. The results suggested that CBS was highly expressed in tumor tissue compared with adjacent non-tumor tissue (P<0.0001) (**Figure 2B**), which confirms that CBS was highly expressed in AEG.

Detailed clinical information for all patients is shown in **Table 1**. Univariate analysis demonstrated that differences in CBS expression were related to the degree of tumor differentiation (P=0.037), TNM staging (P<0.0001), T staging (P=0.008), N staging (P=0.028), and the proportion of recurrences within 5 years (P=0.0034).

CBS expression in tumor tissue was positively correlated with tumor angiogenesis

Angiogenesis is indispensable for tumor growth. Consequently, observing angiogenesis in tumor tissue can significantly affect patient prognosis and necessitate the formulation of alternate therapeutic schemes according to the degree of neovascularization. In this regard, observing angiogenesis in tumor tissue is an important part of clinical IHC. In many clinical applications, CD31 is a common molecular marker used to observe angiogenesis in tumor tissues [21].

In this study, the tumor portions of pathological tissue sections from 214 AEG patients were stained for CD31 to observe tumor angiogenesis. The MVD in tumors was assessed according to different CD31 expression levels, which ultimately revealed the number of capillaries per mm^2 in the tissue. At the same time, MVD data in the context of different CBS expression levels were calculated relative to CBS expression in the control group. As shown in **Figure 3**, this revealed that increased CBS expression was significantly associated with an increased MVD in tumor tissue; the difference in tumor MVD between the CBS negative and CBS high expression groups were statistically significant. Additionally, there was a significant difference in MVD between the low and high CBS expression groups (P<0.0001).

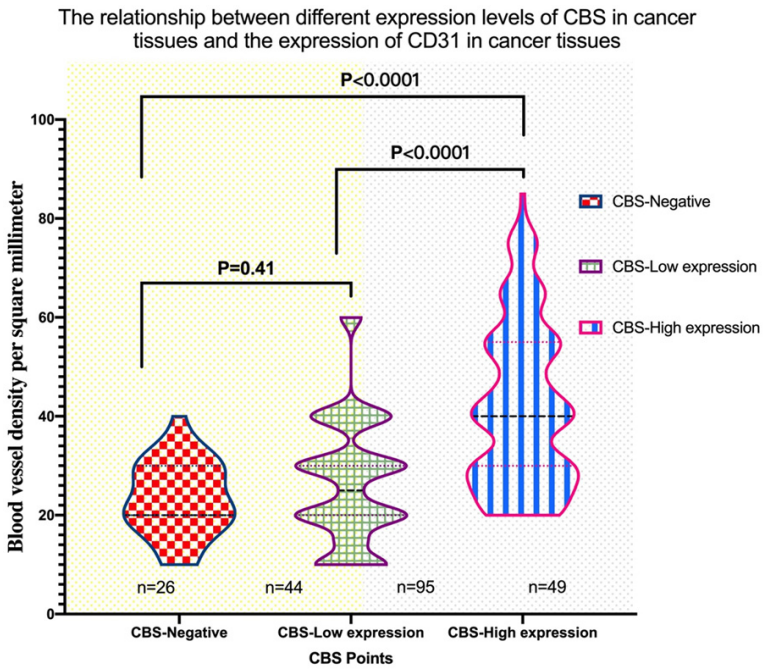

Figure 3. CBS expression in tumor tissue was positively correlated with tumor microvessel density.

As shown in **Figure 3**, tumor MVD increased with increasing CBS expression. Therefore, we analyzed the possible correlation between MVD and CBS expression. Kendall's coefficient was used for this calculation, as these were quantitative data. Our analysis finally obtained *P*=0.0006 and Kendall's coefficient =0.5751. These results indicated that MVD and CBS expression were correlated; specifically, tumor MVD increased with increasing CBS expression.

CBS is a risk factor for the degree of tumor differentiation and 5-year recurrence in AEG patients

Next, we investigated the correlation between CBS and tumor MVD, and the results revealed that CBS expression was associated with increased tumor MVD. Therefore, we examined whether CBS affected the malignant grade of tumors and/or showed different expression levels in different clinical stages.

Univariate analysis suggested that there were significant differences in tumor stage, differentiation degree, and 5-year recurrence between the three groups divided according to CBS expression. Consequently, significant tumor stages, differentiation degrees, and high or low CBS expression cutoff values discovered from multivariate logistic regression analysis were used as the covariates, whereas 5-year recurrence was the dependent variable. With 5-year recurrence, the TNM classification of patients was mostly stage III (relative risk: 1.238; 95% confidence interval [CI]: 1.127-1.444; P< 0.0001), and there was an increased probability of CBS expression (relative risk: 1.639; 95% CI: 1.446-1.915; P=0.015). However, T staging, N staging, and the degree of differentiation had little effect on recurrence in 5 years. This suggested that CBS not only increased tumor MVD, but also increased the probability of progressing to locally advanced disease and/or having a recurrence within 5 years after surgery.

CBS affected DFS in AEG patients

In the previous section, we verified that CBS increased the probability of 5-year recurrence in AEG patients. Therefore, we next summarized DFS among the 214 patients. To clarify the impact of CBS expression on DFS, patients were divided into three groups according to the different levels of CBS expression, including 144 in the high expression group, 44 in the low expression group, and 26 in the negative expression group. The results showed that there was no statistically significant difference in DFS of AEG patients in the CBS-0 (negative) and CBS-1 (low) groups (*P*=0.9), but there were significant differences between the CBS-0 (negative) and CBS-2,3 (high) groups (*P*=0.006), as well as between the CBS-1 (low) groups and CBS-2,3 (high) groups (*P*=0.0002). Thus, increased CBS expression was associated with shorter DFS in AEG patients (**Figure 4**). The median DFS in the high CBS expression group was 46 months, while that in the low expression group was 54 months, which indicates that CBS expression affected the DFS of AEG patients after surgery.

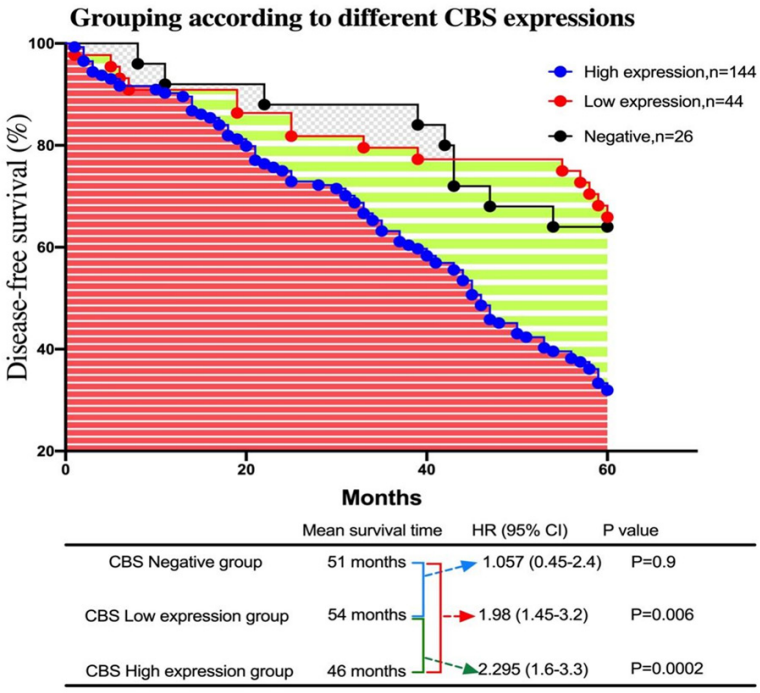

Figure 4. Difference in disease-free survival (DFS) under different levels of CBS expression.

Cox proportional hazards models were then used to quantify the prognostic significance of risk factors after multivariable adjustment. A multivariable analysis was performed to assess the factors that demonstrated significant effects, as in the univariate analysis. After adjusting for competing risk factors, high CBS expression was identified as a risk factor in DFS (hazard ratio [HR]: 1.462; 95% CI: 1.314-1.806; P=0.003). TNM staging was biased towards stage 3 and was associated with an adverse prognosis (HR: 2.605; 95% CI: 1.823-3.735; P<0.0001). Presence of lymph node metastasis (N stage positive) was more likely to affect DFS (HR: 1.620; 95% CI: 1.438-1.877; P=0.007) (**Table 2**).

Discussion

CBS-associated oncogenesis is tumor type-specific. Active CBS expression promotes tumor growth in colon, ovarian, and breast cancers but suppresses tumor growth in glioma. The roles of CBS in liver cancer, gastric cancer, and melanoma remain conflicting and inconclusive [22-24]. The expression of CBS in AEG patients has not yet been reported. In this regard, this study focused on collecting pathological sections from AEG patients to investigate correlations between CBS expression, AEG tumor characteristics, and clinical prognosis. Our results showed that, compared with adjacent non-tumor tissue, CBS expression in AEG tumor tissue was significantly increased, which suggests that CBS plays a key role in the development of AEG tumors.

CBS catalyzes the condensation of Hcy with serine to form cystathionine, which is the initial and rate-limiting step in the transsulfuration pathway. Cystathionine is subsequently cleaved by the enzyme cystathionine gamma-lyase to form cysteine, a precursor of glutathione. Besides this canonical pathway, CBS also participates in the desulfuration reactions that contribute to endogenous H_2S production. The H_2S produced by CBS acts as a small molecular signaling molecule that participates in numerous biological processes, such as regulating inflammation, oxidative stress, and vascular tension [25-28]. In recent years, it was discovered that H_2S is related to tumor cell angiogenesis, invasion, and apoptosis [29].

Additionally, this study explored the relationship between CBS and MVD in tumor tissue. Due to the vessel construction demands of the tumor microenvironment that are needed for tumor growth, tumor cells express VEGFR to produce blood vessels [30, 31]. Then, tumor cells can enter the newly formed tumor vessels and thus the systemic circulation, which is the most important mechanism for distant metastasis. In this regard, MVD can be used to predict prognosis [32]. Using immunohistochemistry, CD31 can mark microvascular endothelial cells, thereby determining the number of newly formed microvessels, which is expressed as MVD (the number of microvessels per mm^2). Our study discovered that MVD increased in tumors with increased tumoral CBS expression. Furthermore, correlation analysis proved the obvious correlation between increased CBS expression and tumor MVD; in other words, CBS expression was associated with increased

Table 2. Multivariate Cox proportional hazard regression analysis of patients' demographic and clinical characteristics and survival (n=214)

Variables	DFS	
	HR (95% CI)	*P* value
CBS High-expression *vs.* Low-expression and Negative expression	1.462 (1.314-1.806)	0.003
HER-2 High-expression *vs.* Low-expression and Negative expression	1.791 (0.983-1.843)	0.078
Sex: Female *vs.* Male	1.071 (0.654-1.452)	0.898
Age ≥ 60 *vs.* <60	1.048 (0.827-1.282)	0.886
TNM staging: III *vs.* I-II	2.605 (1.823-3.735)	<0.0001
T stage: III-IV *vs.* I-II	0.874 (0.656-1.165)	0.358
N stage: Positive *vs.* Negative	1.620 (1.438-1.877)	0.007
Differentiation: Highly differentiated *vs.* Moderately and Poorly differentiated	0.759 (0.684-1.171)	0.073
Smoking history *vs.* non-Smoking history	1.406 (1.958-2.066)	0.082
Drinking history *vs.* non-Drinking history	1.135 (0.871-1.371)	0.264

tumor neovascularization. Previous reports have suggested that CBS can activate pathways such as mitosis-related protein kinase and phosphoinostide-3 kinase to regulate angiogenesis. Additionally, CBS expression can increase H_2S production, which alters the tumor microenvironment [33]. Moreover, H_2S significantly increases the transcription of VEGF, EGF, and PDGF, the phosphorylation of VEGF and PDGF, and upregulates VEGFR and PDGFR protein expression [28], ultimately promoting angiogenesis [34]. This conclusion was validated in a large sample of patients, which verified that CBS directly increased the number of newly formed tumor blood vessels. Thus, detecting CBS expression will provide a foundation for guiding whether AEG patients would benefit from anti-angiogenesis treatments.

CBS can affect tumor angiogenesis, so high or low CBS expression can significantly predict tumor stage and patient prognosis. This study conducted a multivariate logistic regression analysis, which revealed that increased CBS expression could increase the probability of 5-year recurrence. At the same time, univariate analysis showed that increased CBS expression was associated with local late T stage and positive N stage. Finally, we calculated the DFS of patients after surgery, and the results revealed that patients with high CBS expression had markedly shorter DFS, and the difference was statistically significant compared with the low CBS expression group. This indicated that CBS predicted not only tumor stage of AEG patients, but also shorter postoperative DFS in patients with high expression, which was a risk factor in this cohort. Previous studies have also reported pathogenic roles of CBS in cancer; thus, using CBS as a prognostic/predictive biomarker is becoming attractive [35]. Altered CBS levels can also be indicated by changes in Hcy and/or H_2S levels. The potential prognostic values of Hcy in cancer have been extensively studied [36, 37]. Currently, detecting H_2S levels in expired breath or the H_2S degradation level in urine can predict the prognosis of multiple tumors [38]. This provides a foundation for detecting CBS in AEG patients after surgery to determine the best follow-up period and treatment scheme.

Increased understanding of the role of the CBS-regulated networks in cancer biology will significantly promote the development of pharmacological reagents targeting CBS and the identification of appropriate patient populations for these small molecule inhibitors. CBS has important physiological roles in increasing MVD. In future postoperative assessments of AEG patients, CBS can be used as a feasible and practical marker to predict patient prognosis. Additionally, we plan to perform future studies based on these experimental results to determine the relationship between CBS and tumor angiogenesis in AEG and provide a foundation for designing CBS-targeted thera peutics.

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Disclosure of conflict of interest

None.

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