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Cystathionine β-synthase expression correlates with tumor development and poor prognosis in lung squamous cell carcinoma patients

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The purpose of this study was to investigate the correlation between the expression of cystathionine β-synthase (CBS) in lung squamous cell carcinoma (LUSC) and the microvascular density (MVD) and clinicopathological features. Firstly, the expression status of CBS in diffuse carcinoma and LUSC was searched through the public bioinformatics database. Subsequently, immunohistochemical staining and scoring were performed on tumor tissues and matched normal tissues from 108 LUSC patients to assess CBS expression; the MVD of tumor tissues was also detected. The results showed that CBS was overexpressed in some tumor tissues, including LUSC. Immunohistochemical results showed that the positive expression rate of CBS in tumor tissues (63.0%) was higher than that in normal tissues (17.6%). The expression of CBS was correlated with T ($p=0.01$), N ($p=0.004$), TNM ($p=0.011$) stages, and tumor differentiation degrees ($p<0.001$), with the increase of T, N, and TNM stages or the decrease of differentiation, the expression level of CBS also increased. In addition, the expression level of CBS was positively correlated with MVD ($r=0.6997$, $p<0.0001$). Survival analysis showed that the survival rate of the CBS negative expression group was better than that of the positive expression group ($p=0.004$). Cox multivariate analysis showed that CBS expression status ($p<0.001$), T stages ($p=0.020$), and TNM stages ($p=0.021$) were independent factors affecting the prognosis of LUSC. In conclusion, the high expression of CBS affects tumor development and is associated with the poor prognosis of LUCS, which may be used as a biomarker to evaluate prognosis and find a new direction for the treatment of LUSC.

Key words: cystathionine γ-lyase, cystathionine β-synthase, microvessel density, lung squamous cell carcinoma, hydrogen sulfide

Lung cancer is one of the malignant tumors with the highest morbidity and mortality globally, and its incidence has been on the rise in recent years [1, 2]. Non-small cell lung cancer (NSCLC) accounts for the majority of lung cancers. It can be divided into adenocarcinoma, squamous cell carcinoma, adenosquamous cell carcinoma, large cell carcinoma, etc., among which squamous cell lung carcinoma (LUSC) accounts for about 30–40% [3]. For patients with driver positive lung adenocarcinoma (LUAD), targeted drugs such as epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) have achieved good results [4, 5]. However, for LUSC, the breakthrough of targeted therapy has not been achieved, the treatment means are few, and the overall survival rate is low. Therefore, finding a new treatment approach for LUSC has always been a clinical concern.

As a gaseous signaling molecule, endogenous hydrogen sulfide (H_2S) participates in a variety of physiological and pathological processes *in vivo*, and its production requires cystathionineβ-synthase (CBS), cystathionine γ-lysozyme (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST) to decompose L-cysteine [6–10]. In recent years, endogenous H_2S has been found to regulate vascular endothelial cell growth factors (VEGF), promote tumor angiogenesis and metastasis, and participate in regulating the formation and development of a variety of tumors. However, the specific regulatory mechanism has not been determined [11–15]. The tumor microvascular density (MVD) mechanism has always been a critical factor for tumor metastasis [16]. Studies have found that CBS mediated generate endogenous hydrogen sulfide can be triggered by HIF-1α channel to promote the growth and angiogenesis of NSCLC, and tumor MVD also rose accordingly, which resulted in the progression and metastasis of NSCLC [17], but this study is for NSCLC, in the field of pure LUSC, we did not retrieve relevant studies.

CD34 can mark neovascular endothelial cells and is often used to measure tumor MVD [18]. In this study, we first performed a bioinformatics analysis of CBS in an open database and then used immunohistochemical methods to detect the expression of CBS and MVD in LUSC tissues. Finally, the relationship between CBS expression and tumor MVD and malignant biological behavior was evaluated. The results suggest that CBS high expression status is associated with the development and poor prognosis of LUSC, and CBS may be a valuable biomarker.

Patients and methods

Patients and tissue samples. A total of 108 patients with primary LUSC, aged between 46 to 79, who underwent surgical treatment in the Department of Thoracic Surgery of the Fourth Hospital of Hebei Medical University from January 2014 to December 2014, were included in this study, and surgically resected LUSC tissues and adjacent normal tissues were collected. The central area of the tumor tissues and the normal tissues 5 cm away from the tumor edge was selected for marking and sampling, and then a pathological chip with a thickness of 4 μm was prepared. According to the eighth edition of lung cancer staging, all patients were classified for postoperative TNM staging. Clinical information of the patients was collected, including gender, age, smoking history, drinking history, and postoperative pathology. Follow-up was conducted in the Follow-up Center of the Fourth Hospital of Hebei Medical University. The endpoint of follow-up was the death of the patient or the last follow-up time, and the last follow-up time was January 1, 2020. All participants in this study signed informed consent, approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University (2019MEC115).

The expression of CBS in LUSC was evaluated using a public bioinformatics database. Timer (https://cistrome.shinyapps.io/timer/) analysis of CBS mRNA expression in Pan-cancer was used, then UALCAN (http://ualcan.path.uab.edu/analysis.html) was used to evaluate the CBS protein expression differences in lung tumors. Again, through TCGA (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) in LUSC tissue and normal tissue to CBS expression differences for validation. Finally, CBS high and low expression of survival difference was analyzed by Kaplan-Meier (https://kmplot.com/analysis).

Immunohistochemistry and evaluation of CBS and MVD expression. CBS polyclonal antibodies were purchased from Wuhan ABclonal Company (EPR8579) and CD34 polyclonal antibodies were purchased from Fuzhou MaiXin Company (QBEnd/10). The SP method was used for immunohistochemistry experiments [19]. Two pathologists judged the result using a double-blind method to judge independently. When the results were inconsistent, the lower staining score avoided artificial over-judgment and false positives.

CBS staining score. The proportion of positively stained tumor cells and the staining intensity was evaluated and scored independently. The proportion of positive tumor cells is scored as follows: 0 (no stained tumor cells), 1 (stained tumor cells <25%), 2 (stained tumor cells 25–50%), 3 (stained tumor cells 50–75%), 4 (stain 75–100% of tumor cells). Use the following criteria to evaluate the grade of dying intensity: no positive coloring – 0 points, light yellow – 1 point, browns – 2 points, tan – 3 points. The staining index is calculated by multiplying the staining intensity score by the percentage of positive tumor cells. By this evaluation method, the CBS expression score is 0, 1, 2, 3, 4, 6, 8, 9, or 12. A score of <3 is regarded as negative for CBS expression, and a score of ≥3 is regarded as CBS positive expression; 3–4 is divided into low expression; 6–8 is a medium expression, and 9–12 is high expression.

MVD detection was performed using the Weidner method [18], CD34 was used to label tumor microvascular endothelial cells, and the positive was light brown to dark brown. Under the microscope, vascular endothelial cells or clusters of endothelial cells stained brown-yellow and separated from adjacent blood vessels, tumor cells, or other tissues are regarded as capillary. Five dense microvessel areas in the tumor stroma were counted and the average value was regarded as the MVD value.

Statistical analysis. The GraphPad Prism 9.1 software was used for statistical analysis and graph construction. The counting data were expressed in n (%), and the comparison between groups was conducted by the c^2 test, the measurement data were expressed as $\bar{x} \pm s$, and for the comparison between groups, t-test was used. The Kaplan-Meier method was used to evaluate the survival rate of patients, and log-rank tests were used to evaluate the difference in prognosis between groups. Cox multivariate regression analysis was performed after the univariate level determined the prognostic significance. All statistical tests were performed at the bilateral significance level of 0.05.

Results

Analysis of CBS expression based on biological information database. First, the expression state of CBS mRNA in pan-cancer was explored by using the Timer database. The results showed that CBS mRNA was highly expressed in various tumors, including LUSC and LUAD (Figure 1A). Next, the expression of CBS protein in NSCLC was further explored through the UALCAN database. The results showed that CBS protein was overexpressed in LUAD (Figure 1B, $p<0.01$), but CBS protein expression status in LUSC was not retrieved. We further verified that the CBS gene was highly expressed in LUSC through the TCGA database; a total of 502 tumor tissues and 49 normal tissues were retrieved, with a median of 0.095 (0.066–0.129) in the standard group and 0.172 (0.047–0.381) in the tumor group, with statistically significant differences (Figure 1C, $p<0.01$). Finally, the

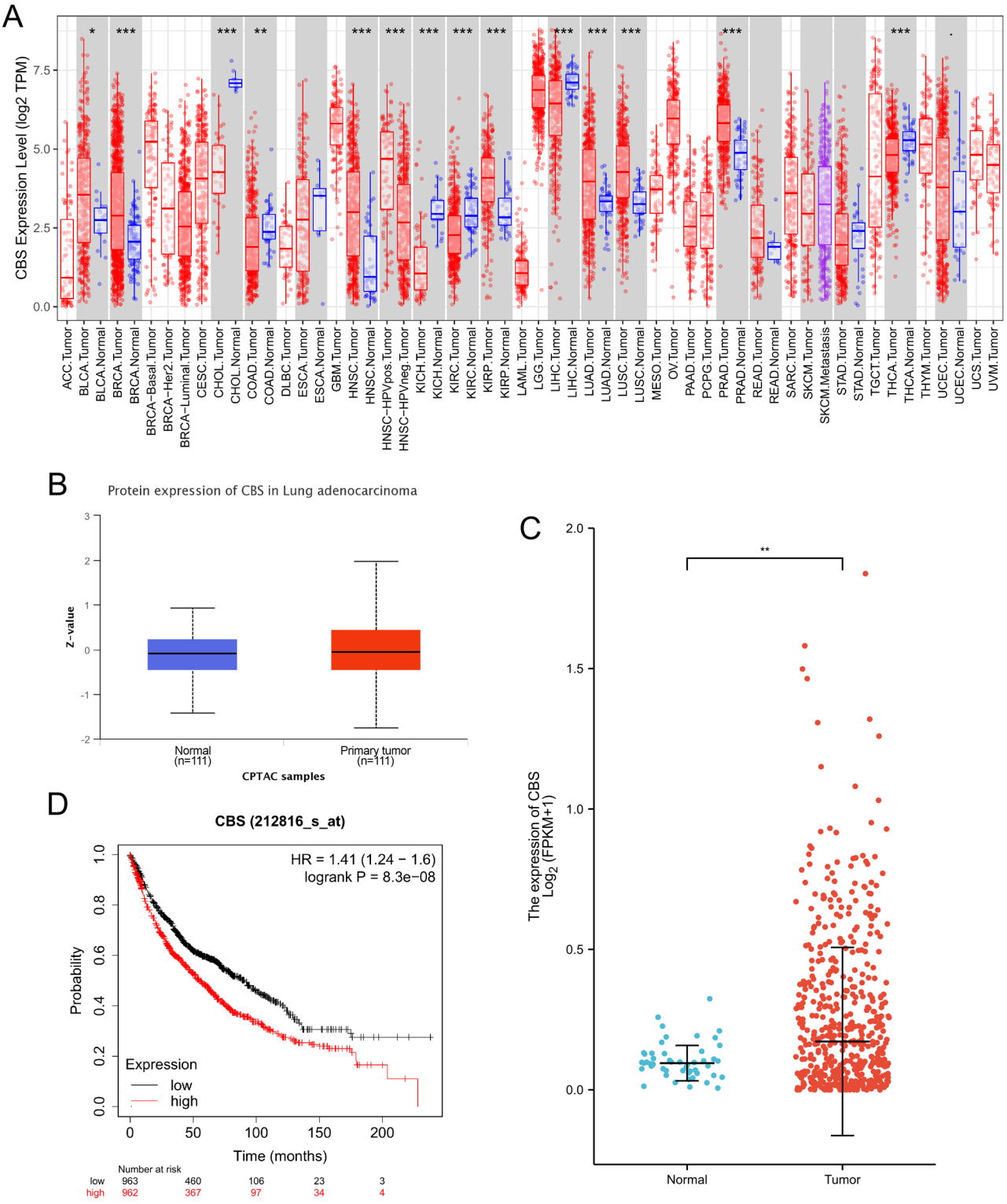

Figure 1. The expression status of CBS in LUSC based on the bioinformatics database. A) The expression level of CBS mRNA in pan tumor and normal tissues based on the Timer database. B) The Expression level of CBS protein in LUAD and normal tissues based on the UALCAN database. The X-axis represents normal tissue or LUAD, and the Y-axis represents CBS protein content, CBS protein was overexpressed in LUAD (p=2.719051e-03). C) The expression level of CBS mRNA in LUSC and normal tissues based on the TCGA database, a dot represents a sample, and the Y-axis represents CBS mRNA content. D) Influence of CBS expression status on survival of lung cancer patients based on the Kaplan-Meier database. ns: p>0.05; *p<0.05; **p<0.01; ***p<0.001

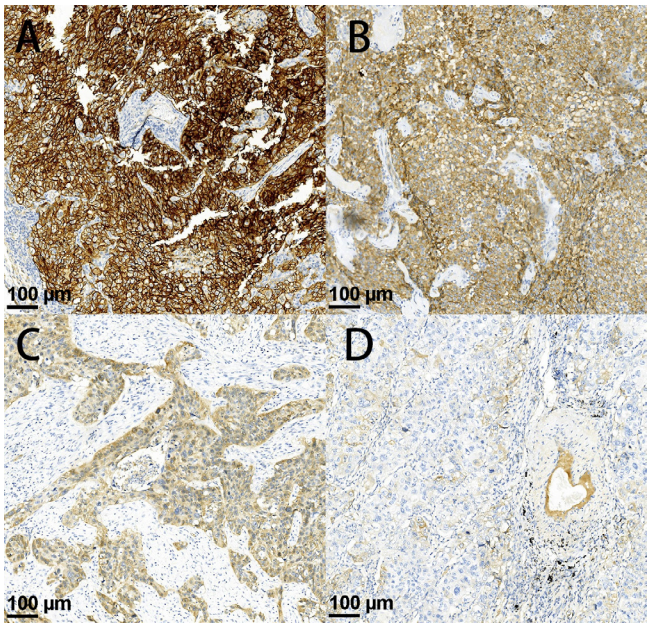

Figure 2. CBS cytoplasmic immunohistochemical staining of LUSC tissues. A–C) CBS positive expression staining of LUSC tissues (A: high expression, B: medium expression, C: low expression). D) CBS negative expression staining of LUSC tissues. Original magnification: A–D, ×200

survival of 2,437 lung cancer patients was analyzed by the Kaplan-Meier database and the survival curve of the CBS high and low expression group was depicted. The results showed that the median survival time of the CBS low expression group was 91 months, and that of the high expression group was 55.37 months, which was statistically significant (Figure 1D, $p<0.05$).

CBS is overexpressed in LUSC. The immunohistochemical method detected the expression of CBS protein in cancer tissues and normal tissues. The results showed many expression states in cytoplasmic staining of cancer tissues (Figures 2A–2D). 68 of 108 cancer tissues were positive (63.0%), the positive expression rate increased with the increase of stage, and the positive rate of stage IV reached 100% (Figures 3A, 3B). 19 of 108 normal tissues showed positive expression (17.6%, Figure 3A), which was statistically different from cancer tissues ($p<0.001$), indicating that the expression of CBS protein was significantly upregulated in LUSC.

The expression of CBS and MVD was positively correlated, and both were correlated with the clinicopathological features of LUSC. There was a positive correlation between the expression levels of CBS and MVD in tumor tissues (Figure 3C, $r=0.6997$, $p<0.0001$). There were no statis-

Table 1. Sample information and expression status of different groups.

Category	n	CBS		χ^2	p-value	MVD	t	p-value
		+	–			$\overline{X} \pm S$		
Sex								
Male	63	40	23	0.018	0.893	39.47±7.34	0.083	0.934
Female	45	28	17			39.60±8.08		
Age								
<60	32	20	12	0.004	0.949	40.03±7.03	–0.443	0.658
≥60	76	48	28			39.31±7.89		
Smoking history								
Yes	46	27	19	0.626	0.428	39.23±7.47	0.337	0.737
No	62	41	21			39.74±7.79		
Drinking history								
Yes	41	27	14	0.237	0.626	40.53±7.77	–1.076	0.284
No	67	41	26			38.91±7.52		
T classification								
T1+T2	61	32	29	6.632	**0.010**	35.19±5.35	8.804	**<0.001**
T3+T4	47	36	11			45.14±6.38		
N classification								
N0	36	16	20	7.941	**0.004**	37.13±7.01	2.350	**0.021**
N1+N2	72	52	20			40.72±7.69		
Differentiation classification								
Low	50	40	10	11.589	**<0.001**	43.30±7.71	–5.353	**<0.001**
Middle+High	58	28	30			36.27±5.89		
TNM classification								
I+II	64	34	30	6.520	**0.011**	35.922±5.926	7.195	**<0.001**
III+IV	44	34	10			44.773±6.768		

tical differences in the expression levels of CBS and MVD in age, sex, smoking history, and drinking history groups (p>0.05), but there were statistical differences in T (p=0.01), N (p=0.004), TNM (p=0.011) stages, and tumor differentiation degrees (p<0.001) groups (Table 1). With the increase of T, N, and TNM stages and the decrease of differentiation degrees, the expression levels of CBS and MVD also increased correspondingly, but not all adjacent groups were statistically different (Figures 4A–4H).

Correlation between CBS expression and prognosis of LUSC. Kaplan-Meier method was used to depict the survival curve of patients. The mean overall survival time was 47.4 months, and the 5-year survival rate was 42%. The prognosis of patients in the CBS negative expression group was better than that in the positive expression group (Figure 3D), and log-rank analysis showed that the difference between the two groups was statistically significant (p=0.004). Univariate analysis of clinical factors that may affect the survival of patients showed that the survival time was related to T, N, TNM stages, and tumor differentiation degrees. These factors were included in the Cox proportional risk regression model. The results showed that T stages (p=0.020), TNM stages (p=0.021), and CBS expression level (p<0.001) were independent factors affecting the prognosis. N stages (p=0.633) and tumor differentiation degrees (p=0.228) did not affect the prognosis (Table 2).

Discussion

CBS is one of the essential coenzymes for endogenous H_2S generation, and its expression status can reflect the activity of H_2S in LUSC. Studies have shown that the expression of CBS and CSE is elevated in colon, ovary, prostate, and other tumor tissues, and the endogenous H_2S involved in the generation can regulate the expression of VEGF in endothelial cells and stimulate tumor microangiogenesis [20–23]. Similar results were obtained by searching the public bio information database (Figures 1A–1D); that is, CBS is highly expressed in some tumors and affects the prognosis, and its mechanism of action needs further study. In terms of LUSC, whether endogenous H_2S affects tumor MVD and prognosis has not been determined yet. Therefore, it is significant to explore the relationship between CSE expression status in LUSC and MVD and clinical prognosis or to find a breakthrough point for the treatment of LUSC.

In this study, it was found that CBS was overexpressed in tumor tissues, and the expression level of CBS increased with the increase in stage, suggesting that CBS-mediated endogenous H_2S may be involved in the proliferation or metastasis of LUSC. Further grouping results showed that not all adjacent groups had statistical differences. That may indicate that the role of CBS in tumor development is gradual and complex. Studies have shown that tumors need to form new blood

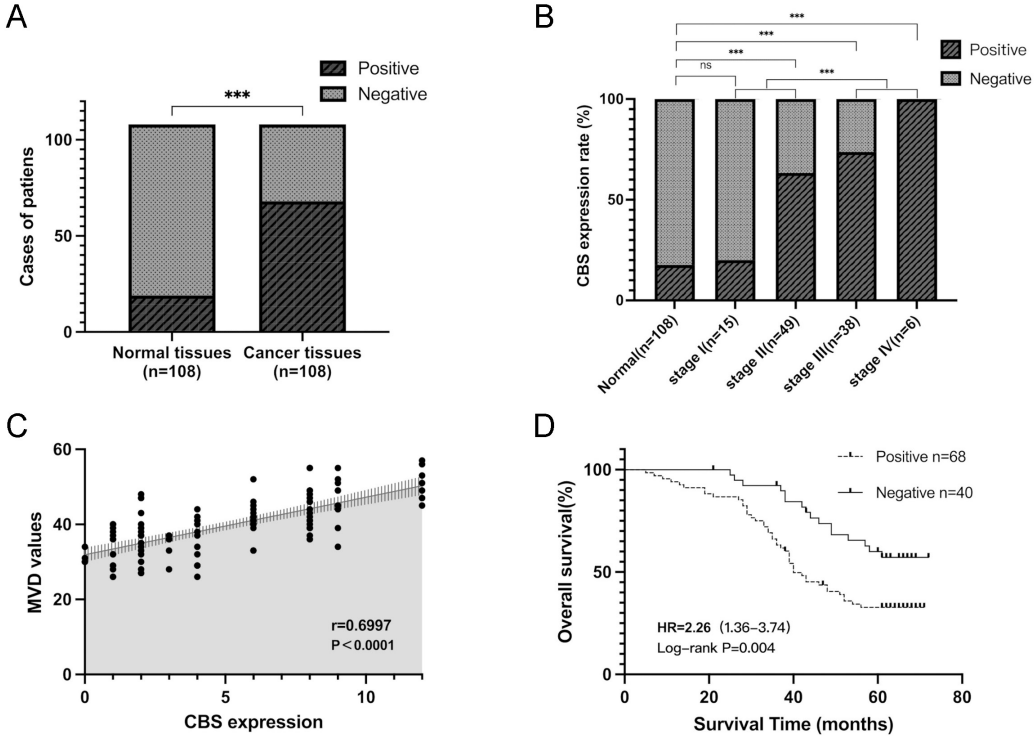

Figure 3. CBS is overexpressed in LUSC tissues and is associated with tumor MVD, which affects LUSC prognosis. A) CBS expression in LUSC and adjacent normal tissues. B) Expression status of distinct LUSC clinical stage and adjacent normal tissues. C) Correlation between CBS and MVD expression in LUSC tissues. A dot represents a sample, with a CBS score on the X-axis and MVD value on the Y-axis. D) Effect of CBS expression level on overall survival. ns: p>0.05; *p<0.05; **p<0.01; ***p<0.001

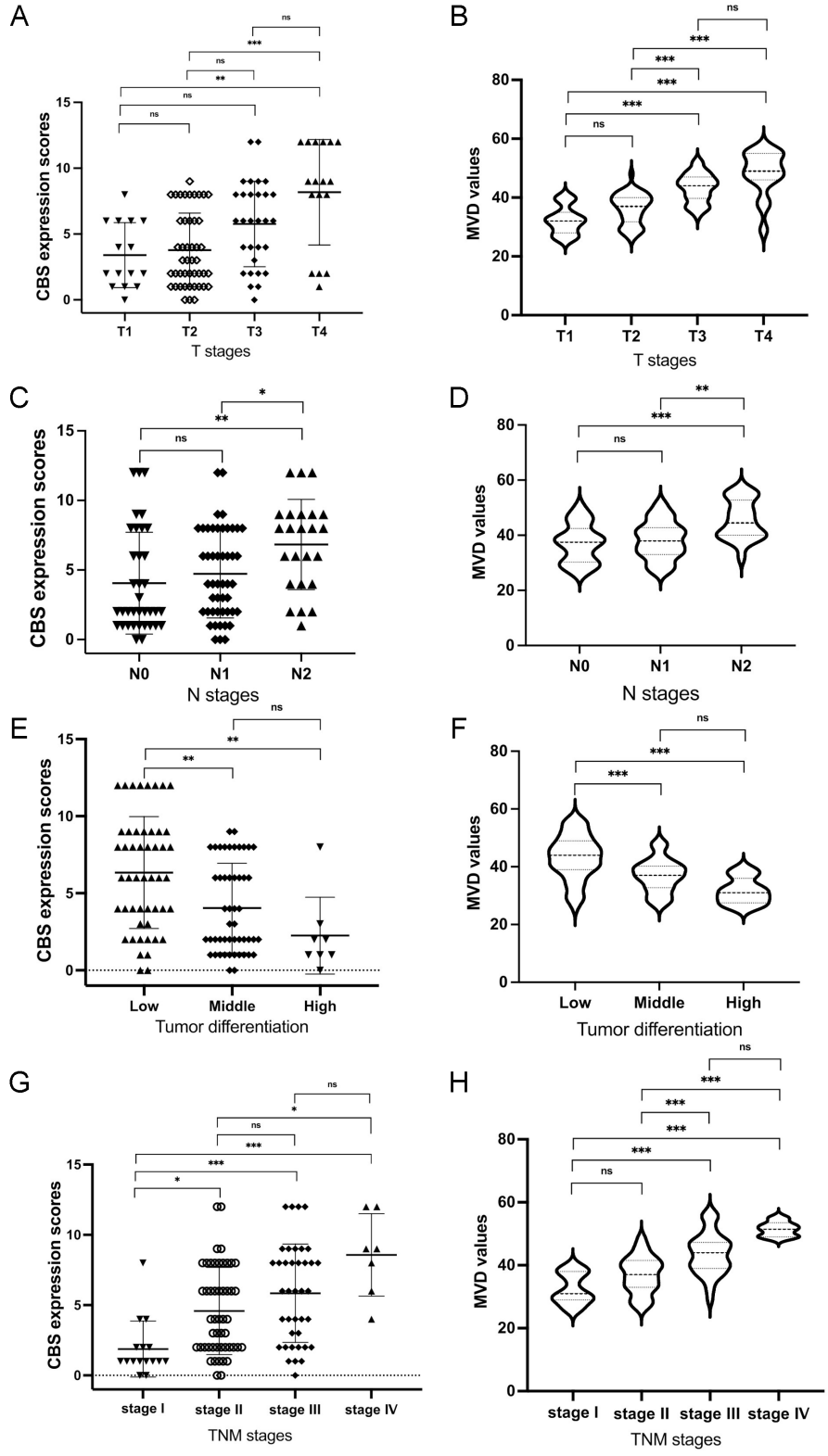

Figure 4. The expression of CBS and MVD in different T, N, TNM stages, and differentiation degrees. In A, C, E, and G, a dot represents a sample, and the Y-axis represents the score of CBS. In B, D, F, and H, the Y-axis represents the expression level of MVD. A, B) Expression status of CBS and MVD in different T staging groups. C, D) Expression status of CBS and MVD in different N staging groups. E, F) Expression status of CBS and MVD in different differentiation groups. G, H) Expression status of CBS and MVD in different TNM staging groups. ns: p>0.05; *p<0.05; **p<0.01; ***p<0.001

Table 2. Univariate and multivariate Cox regression survival analysis of clinicopathological parameters and CBS expression in patients with LUSC.

Parameters	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	p-value	Hazard ratio (95% CI)	p-value
Age	1.001 (0.965–1.038)	0.971		
Smoking history	0.812 (0.484–1.363)	0.430		
Sex	1.255 (0.747–2.107)	0.390		
Drinking history	1.218 (0.728–2.037)	0.452		
T classification	2.886 (2.143–3.885)	**<0.001**	1.723 (1.091–2.719)	**0.020**
N classification	1.991 (1.374–2.885)	**<0.001**	1.131 (0.682–1.878)	0.633
Differentiation classification	0.385 (0.242–0.613)	**<0.001**	1.471 (0.786–2.752)	0.228
TNM classification	4.445 (2.819–7.008)	**<0.001**	2.690 (1.163–6.226)	**0.021**
CBS expression	1.291 (1.187–1.404)	**<0.001**	1.190 (1.092–1.297)	**<0.001**

vessels in the surrounding environment for rapid proliferation and metastasis of tumor cells to deliver nutrients and oxygen to tumors. Therefore, hypoxia is a specific feature of the microenvironment around most solid tumors [24, 25], and H_2S regulates hypoxia *in vivo* to induce tumor angiogenesis [26, 27]. Previous studies have found that endogenous H_2S plays a vital role in promoting the proliferation of breast cancer cells and ovarian cancer cells. It is related to the stage and degree of tumor differentiation [11, 28], which is consistent with the results of our study. In addition, the application of CBS inhibitors in tumor-bearing nude mice can reduce the proliferation and metastasis potential of tumor cells and inhibit angiogenesis and the growth of transplanted tumors [17], which all suggest that CBS is related to the biological behavior of malignant tumors.

This study also found that the expression of MVD was related to the biological behavior of the tumor, and the expression value of MVD increased with an increase in stage or decrease in differentiation degree. The consistent change in MVD and CBS expression suggests that they may be correlated. Although some studies have described the complex interaction between H_2S and VEGF, the specific mechanism of action has not been determined [27, 29, 30]. However, some studies have pointed out that the role of endogenous H_2S in breast tumor metastasis depends on the VEGF signaling pathway, and positively regulates the expression of VEGF and increases the level of some essential proteins in the VEGF pathway [31], which confirms the positive correlation between CBS and MVD in this study. These results suggest that CBS may play a key role in regulating tumor MVD.

The prognosis of patients with high CBS expression is worse, and CBS is an independent factor influencing the prognosis of LUSC; this suggests that CBS may serve as a biomarker to assess the prognosis of LUSC patients, and it is a new idea to treat tumors by blocking the CBS pathway. That has been demonstrated in a study of CSE, another critical enzyme that produces endogenous H_2S. In metastatic breast cancer cells, the application of novel CSE inhibitors can achieve the purpose of anti-tumor cell proliferation and anti-metastasis activity by inhibiting the VEGF signaling pathway [31]. At the same time, other studies have shown that CBS inhibitors can also be combined with anti-tumor drugs to obtain a better therapeutic effect. Inhibiting CBS generation can make LUAD cells sensitive to chemotherapy drugs [32], Sanjib et al. [11] reported that CBS could control cell redox response, regulate mitochondrial bioenergy, promote ovarian tumor growth, and produce drug resistance. CBS may serve as a potential therapeutic target for ovarian cancer recurrence and platinum resistance. These results suggest that CBS or CSE-mediated generation of endogenous H_2S may become the next research direction for tumor therapy, including LUSC.

There are also some shortcomings in this study. Firstly, we only detected the expression of CBS in LUSC, so it is impossible to determine whether there is synergism or mutual exclusion among the three coenzymes that generate endogenous H_2S. The interaction between the three coenzymes is not clear in current relevant studies. In other words, CBS expression status cannot fully represent the activity of endogenous H_2S. Secondly, it has been found that the H_2S donor agent can inhibit the proliferation of tumor cells and promote the apoptosis of hepatocellular carcinoma and gastric carcinoma cells [33, 34], which is contrary to the results of this study. We speculated that different concentrations of H_2S may lead to its bidirectional behavior; Hellmich et al. [13] found that low concentrations of endogenous H_2S tended to promote tumor cell growth, while higher concentrations of H_2S tended to inhibit tumor cell proliferation. Of course, further studies are needed.

To sum up, as far as LUSC is concerned, patients with high CBS expression have a worse prognosis; CBS can be used as a biomarker to predict the prognosis of LUSC.

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