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RESEARCH ARTICLE

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Expression of SET domain bifurcated histone lysine methyltransferase 1 and its clinical prognostic significance in hepatocellular carcinoma

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Abstract

Background: To detect the expression of histone methyltransferase SETDB1 in hepatocellular carcinoma, and to analyze the relationship between SETDB1 expression and tumor size, microvascular invasion, pTNM stage, gender, age, tumor number, tumor differentiation, and other clinicopathological characteristics.

Methods: Immunohistochemical method was used to detect the expression of SETDB1 proteins in liver cancer tissues and adjacent tissues of 100 cases. The qRT-PCR method was used to detect the expression of SETDB1 mRNA in hepatocellular carcinoma and adjacent tissues of 64 cases.

Results: The expression of SETDB1 protein and mRNA in hepatocellular carcinoma was higher than that of adjacent normal liver tissue ($p < 0.05$). High protein expression of SETDB1 was associated with tumor size, MVI presence, and pTNM stage ($p < 0.05$). Univariate analysis revealed that the tumor size, tumor differentiation, MVI grade, and pTNM stage were correlated with DFS, while tumor size, MVI grade, pTNM stage, and SETDB1 protein expression were correlated with OS. Multivariate analysis showed that the combination of MVI grade and pTNM stage has statistical significance in predicting prognosis, while SETDB1 protein expression was not significant prognosis factor.

Conclusions: SETDB1 has a certain role in HCC progression and may act as a prognostic predictor concerning the survival of HCC patients.

KEYWORDS
epigenetics, hepatocellular carcinoma, histone methyltransferase, prognosis, SETDB1

1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in China. Most patients with HCC are diagnosed at an advanced stage and lose the opportunity of surgery or liver transplantation. The overall 5-year survival rate of HCC patients is only about 5%.[1]

Therefore, to find the key genes regulating the occurrence and development of HCC will provide a new strategy for clinical treatment. A large number of researches indicate that transcription

silencing of tumor suppressor genes induced by histone methylation is closely related to the occurrence and development of HCC.[2] SET domain bifurcated histone lysine methyltransferase 1 (SETDB1) is located on chromosome 1 and is involved in the methylation of histone H3K9me3.[3,4] SETDBl is highly expressed in malignant melanoma, prostate cancer, lung cancer, breast cancer, gastric cancer, and colon cancer, which can promote the proliferation, migration, and invasion of tumor cells.[5-10] The expression of SETDBl protein and mRNA in HCC tissues was detected by immunohistochemistry and the qRT-PCR method in our study. The relationship between its expression and the clinicopathological parameters of HCC was analyzed.

2 | MATERIALS AND METHODS

2.1 | Tissue samples

All tissue samples were obtained from Ningbo Diagnostic Pathology Center. Formalin-fixed, paraffin-embedded (FFPE) blocks of HCC tissues ($n = 100$) and normal liver tissues adjacent to the carcinoma ($n = 100$) were acquired from January 2016 to December 2018. The criteria for included cases were as follows: (1) Pathologically diagnosed HCC, (2) not received TACE, radiofrequency, radiotherapy, and targeted drugs before operation. Exclusion criteria: (1) carcinoma is mixed HCC and other types of liver cancer, (2) with a history of other malignant tumors. All cases were reassessed according to the WHO Classification of Tumors 5th Edition by two liver pathologists. The clinicopathological parameters were obtained from electronic medical records and pathological diagnosis reports. The study was approved by the ethics committee of Ningbo Diagnostic Pathology Center. Follow-up began 1 month after surgery by telephone or outpatient review, included laboratory examination and abdominal B ultrasound or CT/MRI. The follow-up period ended until October 31, 2020, and the median follow-up period was 34 (12–52) months.

2.2 | RNA extraction and real-time fluorescence quantitative PCR

FFPE RNA extraction kit (OMEGA bio-tek) was used. SETDBl primers were designed by Primer 5.0 software and synthesized by Shanghai Bioengineering Company. The primers of the SETDB1 gene were as follows: 5′-CTATATGACTTCCGGCGGATGA-3′ (forward) and 5′-GCATTGTCCGAAGGCAGAGA-3′ (reverse). The housekeeping gene GAPDH was utilized as an endogenous control. Total RNA was extracted from 64 cases of HCC and paracancer tissues according to the instructions. A260/A280 ratio from 1.8 to 2.1 was the criteria for RNA extraction. cDNA was synthesized by Luna Universal Probe One-step qRT-PCR Kit (NEW ENGLAND BioLabs). PCR cycle conditions were as follows: 95℃ for 1 min, then 95℃ for 10 s (40 cycles), and finally 60℃ for a 1-minute extension. Each sample was repeated three times. The comparative cycle threshold (CT) ($2^{-\Delta\Delta Ct}$) method was used to calculate SETDB1 relative gene expression.

2.3 | Immunohistochemistry (IHC)

The FFPE blocks were collected and 5.0-m paraffin sections were used for IHC. IHC was performed according to the antibody instructions by Envision two-step method. Rabbit anti-human polyclonal antibody SETDB1 (Proteintech Group) was diluted at 1:200. The positive control referred to the reagent instructions, and the negative control used PBS instead of primary antibody. The positive signal was localized to the nucleus. The scoring rules of SETDB1 IHC results refer to Shen X, et al.[11] Less than 3 is divided into negative, greater than or equal to 3 is positive.

2.4 | Statistical analysis

The SPSS 17.0 software was used to analyze the data. Measurement data was listed as the mean ± standard deviation. The differences between the numerical data were tested by Student's *t* test. Categorical data were listed as rate (%). A chi-square test was used to compare the expression of SETDB1 protein in liver cancer tissues and adjacent normal liver tissues, as well as the relationship between SETDBB1 expression and clinicopathological features. $p < 0.05$ was considered statistically significant. Survival curves were plotted by the Kaplan-Meier method, and the log-rank test compared the survival differences in different groups. The correlation between the clinical characteristics of HCC and SETDB1 expression was evaluated by Cox proportional hazard model. $p < 0.05$ showed a statistically significant difference.

3 | RESULTS

3.1 | SETDB1 expression levels in HCC tissues and normal liver tissues adjacent to the carcinoma

We investigated whether SETDB1 expression was different in HCC tissues and normal liver tissues adjacent to cancer. A total of 100 HCC patients were examined. SETDB1 was stained in the nucleus of cells (Figure 1). The positive proportion of SETDB1 was 63.0% (63/100) in HCC tumor tissues, while the number was 11.0% (11/100) in normal liver tissues adjacent to the carcinoma (Figure 2). Most of the 11 normal liver tissues were weakly to moderately positive, and only two were focally positive. The difference of SETDB1-positive expression between HCC and normal liver tissues adjacent to the carcinoma was statistically significant ($p = 0.009$, Table 1).

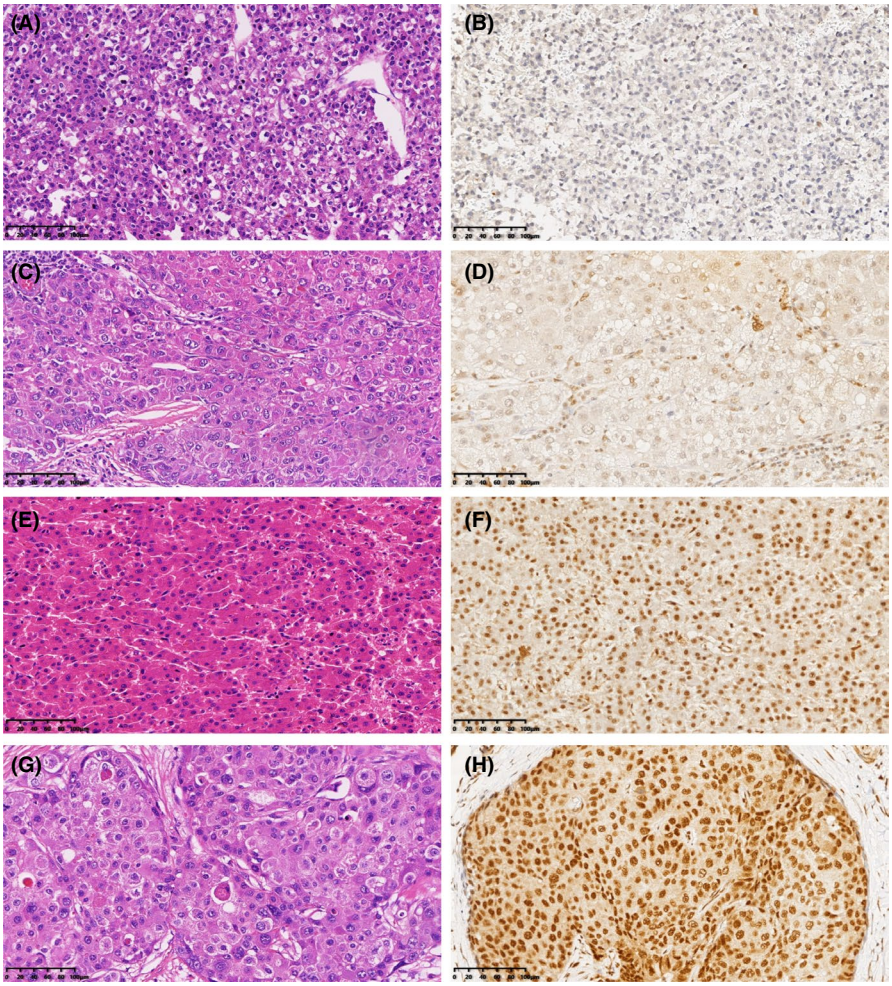


FIGURE 1 Representative immunostaining of SETDB1 in HCC tissues (EnVision). (A) HE stain of HCC tissue; (B) negative expression in case A; HE stain of HCC tissue; (D) weak positive expression in case C; (E) HE stain of HCC tissue; (F) moderate positive expression in case E; (G) HE stain of HCC tissue; (H) strong positive expression in case G. HCC, hepatocellular carcinoma; HE, hematoxylin and eosin

3.2 | Relationship between SETDBl expression and clinicopathological parameters of HCC patients

The statistics showed that SETDBl expression levels in HCC tissues were related to tumor size, MVI grade, and pTNM stage ($p = 0.003$, 0.004, and $p = 0.025$, respectively. Table 2). There was no significant correlation between the expression of SETDB1 protein and the clinicopathological features such as the number of tumors, age, tumor differentiation, and liver cirrhosis ($p > 0.05$, Table 2).

3.3 | SETDBl mRNA level in HCC and relationship between the level and clinicopathological parameters of HCC patients

We detected SETDB1 mRNA levels and analyzed the differences between HCC and adjacent normal liver tissues in 64 HCC patients. Statistics showed the average relative expression of SETDB1 in HCC tissues and normal liver tissues adjacent to the carcinoma was 1.658 ± 1.287 and 1.233 ± 1.070, respectively. The results manifested that SETDB1 mRNA expression was significantly higher in HCC tissues than that in normal liver tissues adjacent to the carcinoma ($t = 2.917$, $p = 0.005$) (Figure 3). The statistics showed that SETDBl mRNA levels in HCC tissues were also related to tumor size, MVI grade, and pTNM stage ($p = 0.024$, 0.025, and 0.029, respectively), which had been published.[12]

3.4 | Correlation between expression of SETDB1 and prognosis of patients

Kaplan-Meier analysis was performed to determine the correlation between SETDB1 protein expression and disease-free survival (DFS), or overall survival (OS) in HCC. We found that patients with positive expression of SETDB1 protein had a shorter OS than those with negative expression ($p = 0.015$, Figure 4A), while the expression of SETDB1 had no effect on DFS. ($p = 0.084$, Figure 4B). Univariate analysis revealed that the tumor size, tumor differentiation, MVI grade, and pTNM stage were correlated with DFS (Table 3), while tumor size, MVI grade, pTNM stage, and SETDB1 protein expression were correlated with OS (Table 3). Multivariate analysis showed that MVI grade and pTNM stage had statistical significance on prognosis (Table 4), while SETDB1 protein expression was not significant prognosis factors.

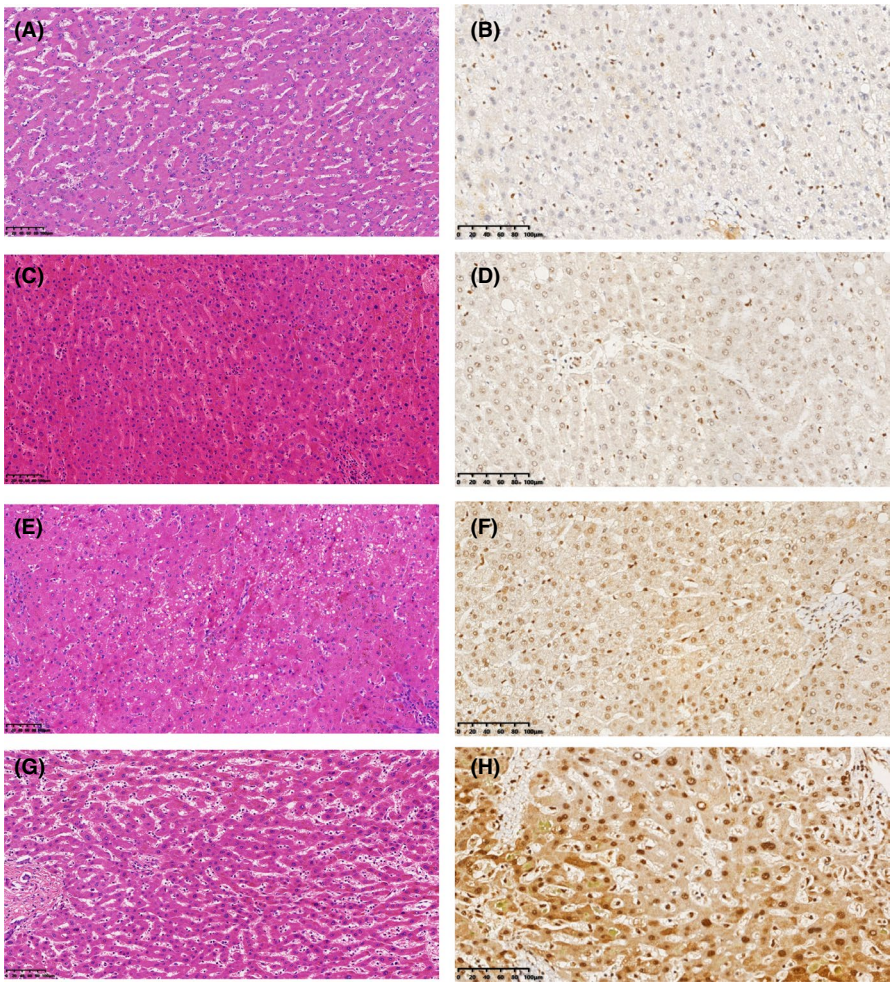


FIGURE 2 Representative immunostaining of SETDB1 in adjacent noncancerous tissues (EnVision). (A) HE stain of normal liver tissue; (B) negative expression in case A; (C) HE stain of normal liver tissue; (D) weak positive expression in case C; (E) HE stain of normal liver tissue; (F) moderate positive expression in case E; (G) HE stain of normal liver tissue; (H) strong positive expression in case G. HE, hematoxylin and eosin

TABLE 1 Comparison of SETDB1 protein expression in HCC tissues and adjacent noncancerous tissues

		SETDB1 expression			
Sample types	NO.	Positive (%)	Negative (%)	χ^2	*p*
HCC	100	63	37	6.76	0.009
adjacent noncancerous tissues	100	11	89		

Abbreviation: HCC, Hepatocellular carcinoma.

4 | DISCUSSION

Epigenetics is one of the hotspots in cancer research. Histone methylation plays an important role in tumorigenesis, development, recurrence, and metastasis. Recent studies have found that SETDB1 expression in liver cancer was significantly elevated, related to the progression, invasion, and prognosis of liver cancer. SETDBl was found to be the most significantly upregulated epigenetic regulator in HBV-related HCC by transcriptome sequencing technology.[13] The up-regulation mechanism may involve multiple steps, including copy number increasing of SETDB1 genes, SETDB1 activity promoted by the SP1 transcription factor. SETDB1 is the direct target gene for the miR-621 and miR-621, enhanced the radiosensitivity of HCC cells via activating the p53-signaling pathway.[14] SETDB1 promotes the proliferation and migration of cells by forming SETDB1-Tiam1 compounds.[15] A study on apoptosis of hepatocellular carcinoma by knocking out SETDB1 showed that downregulation of SETDB1 played anti-HCC cell growth through methylation of H3K9 sites.[16] The main objective of our study was to explore the relationship between SETDB1 expression and clinicopathological parameters of HCC. In our study, we demonstrated that the expression of SETDB1 was associated with some clinicopathological parameters of HCC and speculated that the overexpression of SETDB1 might lead to the development of HCC in the Chinese race.

In our study, we first detected 64 cases of HCC and normal liver tissues adjacent to the carcinoma by RT-PCR. The results showed that the expression level of SETDB1 mRNA in HCC was significantly higher than that in normal liver tissue adjacent to carcinoma. We examined SETDB1 protein expression in 100 HCC tissues and normal

TABLE 2 Relationship between SETDB1 protein expression and HCC patient clinicopathological parameters

Clinicopathological parameters	NO.	SETDB1 expression		χ^2	*p*
		Negative (37)	Positive (63)		
Gender					
Male	80	27	53	1.794	0.180
Female	20	10	10		
Age (year)					
<=60	58	21	37	0.037	0.848
>60	42	16	26		
Tumor size (cm)					
<=5	75	34	41	8.848	0.003
>5	25	3	22		
Tumor number					
1	84	32	52	0.267	0.605
>=2	16	5	11		
Tumor differentiation					
Well-moderate	63	26	37	1.318	0.251
Poor	37	11	26		
liver cirrhosis					
With	60	21	39	0.255	0.614
Without	40	16	24		
MVI grade					
M0	35	20	15	10.959	0.004
M1	49	15	34		
M2	16	2	14		
pTNM stage					
I + II	66	36	30	8.194	0.004
III + IV	34	19	15		

Abbreviation: HCC, Hepatocellular carcinoma.

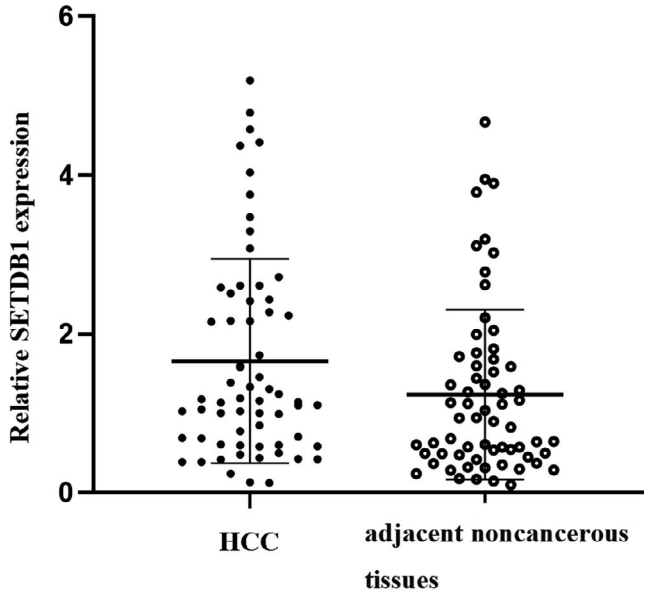
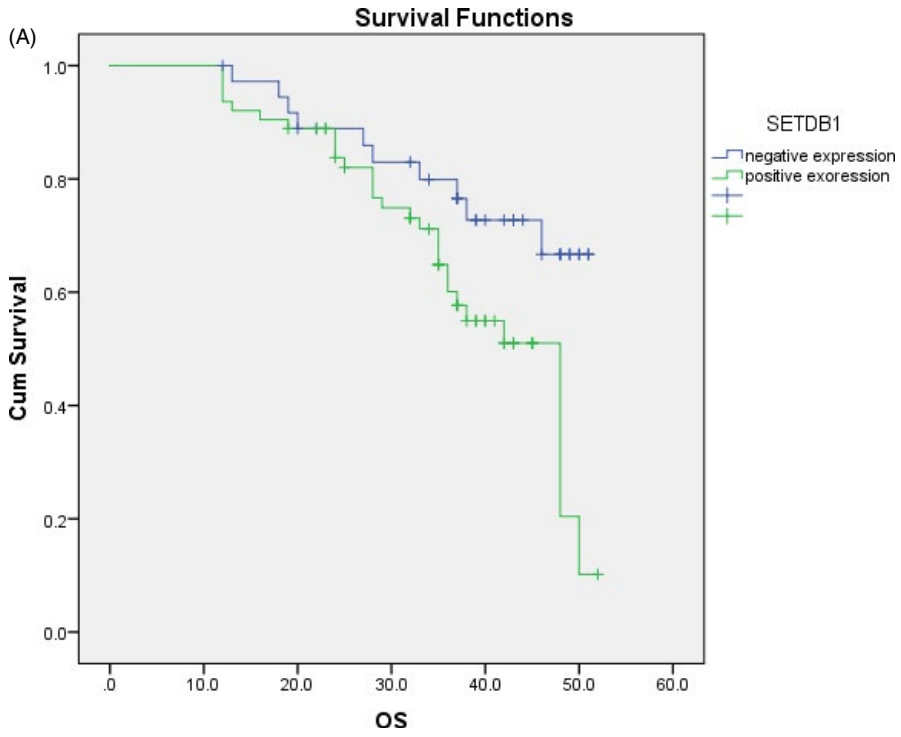


FIGURE 3 SETDB1 was significantly highly expressed in HCC tissues than in adjacent noncancerous tissues ($n = 64$, $t = 2.917$, $p = 0.005$). HCC, Hepatocellular carcinoma

liver tissues adjacent to the carcinoma by IHC. The proportion of high SETDB1 expression in HCC was significantly higher than that in the normal liver tissues adjacent to the carcinoma ($p = 0.009$). These results suggest that the level of SETDB1 expression may be related to HCC occurrence and progress. Furthermore, we evaluated the relationship between SETDB1 protein expression and clinicopathological parameters. The results showed that the high expression of SETDB1 in HCC was correlated with tumor size, MVI, and pTNM stage ($p = 0.003$, 0.004, and $p = 0.025$, respectively), while with no significant correlation with gender, age, number of tumors, tumor differentiation, and associated with cirrhosis. Chen Jing et al. also found that high expression of SETDB1 protein in HCC was associated with tumor size,[16] similar to our study. SETDBl expression associated with MVI, pTNM stage, and tumor size suggest that up-regulation in SETDBl expression may be associated with poor prognosis in HCC patients, and the detection of SETDB1 expression levels in HCC has some value in evaluating clinical tumor progression and patient prognosis.

This experiment used immunohistochemistry to detect the expression of SETDBl protein in 100 primary cancer tissues in HCC. In the subsequent RT-PCR experiment, since the total RNA extraction



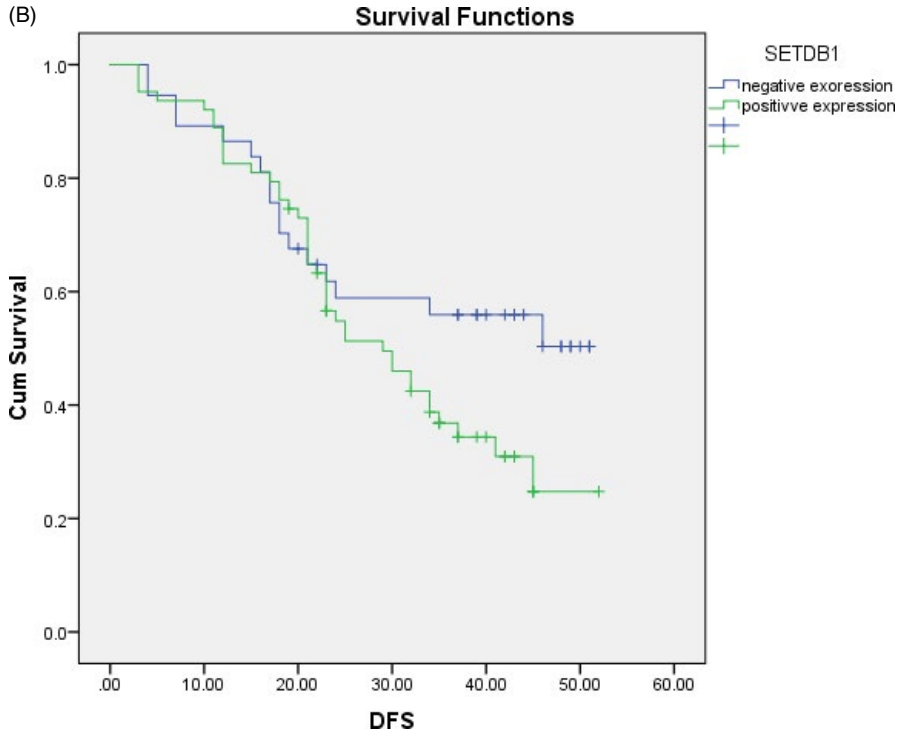


FIGURE 4 The expression of SETDB1 had no effect on DFS and OS. (A) Relationship between SETDB1 protein expression and OS. (B) Relationship between SETDB1 protein expression and DFS. HCC, Hepatocellular carcinoma; DFS, disease-free survival; OS, overall survival

effect of some cases is not ideal, only 64 cases were tested. This experiment used FFPE blocks of HCC tissues. Fresh HCC tissues and HCC cell lines can be selected to further analyze the relevant mechanism of SETDB1 in the development of HCC.

We found that the OS of patients with positive SETDB1 protein expression was shorter than that of patients with negative expression. Univariate analysis revealed that the SETDB1 protein positive expression was correlated with OS. These results suggest that SETDB1 has a certain role in HCC progression. SETDB1 protein may be a significant prognosis factor.

SETDB1 is considered to be a major regulator in many crucial cellular functions.[17] Database analysis found that low SETDB1 expression was associated with poor survival in a variety of tumors, including colorectal cancer, endometrial cancer, thyroid cancer, liver cancer, kidney cancer and melanoma, which is consistent with our research. SETDB1, on the one hand, down-regulates important

TABLE 3 Univariate Cox regression model for the survival of HCC patients

Clinicopathological parameters	DFS		OS	
	χ^2	*p*	χ^2	*p*
Age	1.699	0.192	0.713	0.398
Gender	1.635	0.201	1.193	0.275
Tumor number	0.533	0.465	1.474	0.225
Tumor size	2.229	**0.003**	3.297	**<0.001**
Tumor differentiation	1.736	**0.037**	2.768	0.096
liver cirrhosis	0.549	0.459	0.001	0.973
MVI grade	2.179	**<0.001**	2.618	**<0.001**
pTNM stage	2.622	**<0.001**	3.469	**<0.001**
SETDB1 expression	2.892	0.089	2.391	**0.020**

Note: All statistical tests were two-sided.

Abbreviations: DFS, disease-free survival; HCC, Hepatocellular carcinoma; OS, overall survival.

TABLE 4 Multivariate Cox regression model for the survival of HCC patients

Clinicopathological parameters		95% CI for Exp(B)			
		Exp(B)	lower	Upper	*p*
MVI grade	DFS	2.093	1.245	3.52	**0.005**
	OS	1.924	1.28	2.894	**0.002**
pTNM stage	DFS	2.467	1.257	4.876	**0.009**
	OS	2.071	1.211	3.541	**0.008**

Note: All statistical tests were two-sided.

Abbreviations: CI, confidence interval; DFS, disease-free survival; Exp(B) = OR, odds ratio; OS, overall survival.

tumor suppressor genes through histone methylation, and on the other hand, inhibits tumor-intrinsic immunogenicity, enabling cancer cells to evade immune responses.[18]

5 | CONCLUSION

SETDB1 levels were elevated in primary HCC and were associated with tumor size, MVI and pTNM stages, suggested that SETDB1 was a prognostic indicator of HCC. Although it has been reported that SETDB1 is closely related to the progression, aggression, and prognosis of HCC disease, its specific mechanism in HCC also requires further research in vitro experimental combined and animal experiments. SETDB1 is expected to be a new therapeutic target for HCC. The intracellular mechanisms of SETDB1 and the complex interactions of SETDB1 with other methyltransferases and deacetylases need to be further elucidated.[19] It is necessary to explore research on SETDB1-targeted therapy.

CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the primary author upon reasonable request.

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