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Correlation and clinical significance of methyltransferases SETDB1 and SPG20 methylation in lung adenocarcinoma

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Research

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ABSTRACT

Objective: The incidence of primary lung adenocarcinoma is increasing year by year in worldwide, and it has surpassed squamous cell carcinoma to become the most common pathological type of lung cancer. Advances in medical technology have made the overall survival rate of lung cancer patients improve, but it is still very low. In the study, we investigated the methyltransferase SETDB1 expression and the methylation level of SPG20, and analyzed the correlation and its clinical significance in the pathogenesis of lung adenocarcinoma.

Methods: 60 lung adenocarcinoma and normal lung tissues were selected.The expression of SPG20 and SETDB1 mRNA was examined by RT-qPCR; Protein expression of SPG20 and SETDB1 was determined by immunohistochemistry(SP) and immune protein imprinting(Western blot);Methylation level of SPG20 gene was determined by pyrosequencing. Correlation between SETDB1 and SPG20 methylation levels and both and clinical case characteristics was analysed by statistics. Western blot detection of SETDB1 and SPG20 protein expression in lung adenocarcinoma cells and normal bronchial mucosal epithelial cells.The small interfering RNA of SETDB1 was transfected into lung adenocarcinoma A549 cells by liposome-mediated method, the mRNA and protein expression levels of SETDB1 were detected by RT-qPCR and Wesrern blot, and the methylation level of SPG20 gene was detected by pyrosequencing.

Results: Relative expression of SPG20 mRNA was significantly lower in lung adenocarcinoma tissue than in normal lung tissue, while relative SETDB1 mRNA expression was higher in cancer tissue than in normal lung tissue. SETDB1 protein expression was significantly higher in lung adenocarcinoma tissue than in normal lung tissue, while SPG20 protein showed lower expression in cancer tissue than in normal lung tissue. The methylation rate of the SPG20 gene was significantly higher in cancerous tissues than in normal lung tissue, the difference was statistically significant. SPG20 methylation in cancer tissues showed a correlation with SETDB1 and showed a positive correlation; SPG20 methylation and SETDB1 are closely related with TNM stage, tissue differentiation, and lymph node metastasis in lung adenocarcinoma. The vitro experiments showed that SETDB1 was highly expressed, while SPG20 was lowIn lung adenocarcinoma cells, SETDB1 was low and SPG20 was high in normal bronchial epithelial cells. RNA interference with SETDB1 could significantly reduce the mRNA and protein expression of SETDB1 in A549 cells, the methylation rate of SPG20 gene was significantly decreased, and the protein expression was increased.

Conclusion: There is a significant correlation between methyltransferases SETDB1 and SPG20 methylation in lung adenocarcinoma, and high SETDB1 expression may be the upstream molecule of SPG20 methylation, working together in promoting the occurrence and development of lung adenocarcinoma.

Key words: Methyltransferase; SETDB1; SPG20; methylation modification; lung adenocarcinoma

1.INTRODUCTION

Lung cancer is a common respiratory malignant tumor in the world, among which the patients with lung adenocarcinoma are increasing year by year, and now it has become the most common pathological type of lung cancer[1] [2]. The traditional treatment of lung adenocarcinoma mainly focuses on surgery and postoperative chemoradiotherapy. With the development of medicine, the molecular targeted therapy and immunotherapy of modern precision medicine have made great progress in the new treatment of lung adenocarcinoma[3].Spastic paraplegia-20 (SPG20) encodes a Spartin multifunctional protein, and its aberrant role in the cell division cycle is associated with tumorigenesis[4] [5] [6]. The methylation modification of SPG20 was found to be closely related with the proliferation, invasion and metastasis of various solid tumors. SET domain branch 1(SET domain bifurcated1, SETDBl) is an important family of histone methyltransferases, its function can catalyze the expression of a variety of genes repressed genes, which is closely related to a variety of tumors[7] [8]. It is rarely studied on the association of SETDB1 and SPG20 in the pathogenesis of lung adenocarcinoma at home and abroad。For that reason，we were planing to investigat the SETDB1 and SPG20 methylation levels in lung adenocarcinoma tissues，and explore the relevance of both patients and clinical significancein during the development of lung adenocarcinoma.

2. MATERIALS AND METHODS

2.1. Specimen and cell Lines

Tissue specimens of 60 patients with primary lung adenocarcinoma were treated in the thoracic surgery department of the Affiliated Hospital of Chengde Medical University were from January 2020 to January 2021.Inclusion criteria:Postoperative pathology confirmed lung adenocarcinoma patients with no history of chemoradiotherapy, immunization and gene-targeted therapy before surgery. Experimental group: lung adenocarcinoma tissue; control group: normal tissue (more than 6 cm from the edge of cancerous tissue).

Among them, 16 examples were male, 44 examples were female; ≥60 ages were 22 examples, <60 ages were 38 examples; 17 examples were smoking patients, and 43 examples were non-smokers;TNM staging [9] according to the standards set by IASLC version 8: I stage were 26 examples, Ⅱ stage were 16 examples, Ⅲ stage were 18 examples; 8 patients were poorly differentiated, 38 patients were moderately differentiated, and 14 patients were highly differentiated; 16 examples have lymphatic metastasis, 44 examples have none lymphatic metastasis.This study was approved by the Ethics Committee of the Affiliated Hospital of Chengde Medical University, and informed consent was obtained from the patients. Lung adenocarcinoma cells A549 was provided by the Central Laboratory of the Affiliated Hospital of Chengde Medical University.

2.2. Main reagent

Methylation modification kit was purchased from QIAGEN company（Germany）; pyrosequencing reagent from CST company（USA）; DNA extraction kit from Wuhan Spei Biotech Co.（China）, IHC kit and DAB color development kit from Zhongshan Jinqiao Biological Co., Ltd.（Beijing, China）; SETDB1 and SPG20 monoclonal antibody were purchased from CST Company （USA）.

2.3 Real-Time PCR

The RNA extraction kit extracted total tissue and cellular RNA. 300 ng RNA were applied to PrimeScriptTM RT Reagent kit and reverse transcribed according to the instructions. The expression of SPG20 and DNMT3b mRNA was detected using the SYBR Green qPCR Master fluorescence quantification kit, and the respective lead sequences are shown in Table 1. Relative expression level was calculated as $2^{-\Delta\Delta Ct}$.

2.4. Immunohistochemistry

Tissues were sliced, xylene waxing, soaked in gradient alcohol, driplet with 3%H_2O_2 and placed at room temperature for 10min. Citrate buffer high temperature antigen repair after membrane breaking; Slice cooling then closed with goat serum . After washing, primary antibodies were placed in a wet box and in-

cubated for 4℃ overnight. Secondary antibodies were incubated at room temperature for 30min. DBA coloration, then counterstained with hematoxylin.

2.5. Western blot

The total protein was extracted, and the protein concentration was determined; the total protein was separated by gel electrophoresis, and the total protein was transferred to PVDF membrane by semi-dry transfer method. After blocking with 5% nonfat milk powder at room temperature for 1 h, the membrane was incubated in the diluted primary antibody for 40 min. After rinsing, the membrane was incubated in the diluted secondary antibody for 30 min. After rinsing, the luminescence reaction was performed in a dark room and exposed to film. Image pro plus software was used to analyze the IOD value of the integrated optical density of the protein bands, and the ratio of the IOD value of the target protein to the IOD value of the internal reference was used to reflect the expression level of the target protein.

2.6. Pyrosequencing method

Total DNA was extracted by the DNA extraction kit.The DNA was sulfite-transformed by ED DNA Methylation-Gold kit.The amplified products were subjected to pyrosequencing and methylation rates were automatically analyzing methylation status at each site using PyroQ-CpG software.The forward primer sequence of SPG20 gene was

5′ -AGGAAGTATGAAAGAATGTATTGTAAAG-3′ ,the reverse primer was

5′ -CCCCTCAAAATTAAACAACCTTTCTCTACA-3.

2.7. Cells culture

Well-growing esophageal cancer A549 cells were selected, and the culture flask was gently shaken several times, suspended with debris floating on the cell surface, and then poured out together with the growth fluid and washed once with Hanks solution. Add 0.25% trypsin solution or trypsin-one EDTA digestion solution for 5ml from the cell-free face side, and flip the culture flask to immerse the digestion solution in the cells for about 1min. Flip the culture bottle and place it for 10min. In order to promote cell digestion, 37℃ of pre-warmed digestive solution can be added, or use the palm of the hand to the outer wall of the bottle with the cell surface, until the cell surface is observed by the naked eye. Pour out the digestive juice, and wash with 4.5ml of Hanks liquid along the opposite side of the cell layer. When washing, then turn the culture bottle gently, let the liquid flow slowly in the bottle, so as to wash off the digestive juice such as trypsin and digest after pouring off the trypsin. Add a right amount of newly prepared growth solution along the cell surface, wash the cells, and blow them several times to disperse the cells, and pass the culture at 1:2 or 1:3. In 37℃ culture, it can be adherent for about 30min after inoculation. The growth liquid can be changed for 48 hours. Generally, a monolayer can be formed for 3~4 days. The monolayer is formed, and then the maintenance liquid can be changed for testing.

2.8. Transfection

Log-growth cells were taken and 0.25% trypsin digested to make cell suspension. After counting, cells were performed, seeded into 6-well culture plates, about 1×10^5 cells per well, adding F12 medium containing 10% FBS, and cells were incubated in a conventional incubator. Transfection was performed when the cell density reached 80% per well. After the cells were washed twice with serum-free F12 medium on the day of transfection, 1.5ml of serum-free F12 medium was added to each well and further starved into a cell incubation box for 4h before transfection. Dilute 1.25 μl 20μM siRNA of storage liquid (V2) with 30μl 1× riboFECT™ CP Buffer (V1), Gently mix well, Room temperature was incubated for 5min; Add the 3 μl riboFECT™ CP Reagent (V3), Gently blow well and mix well, Room temperature was incubated for 15min; The riboFECT™ CP mixture was added to 465.75 μl of cell culture medium (V4), Bring the total volume up to 500 μl, Gently mix well; The culture plates were incubated in a 37 ℃ CO_2 incubation tank for 48h.

2.9. Statistical Analysis

Data of the two comparing groups were analyzed by the Student's t-test using the SPSS, version 21.0. Measurement data are expressed by the mean ± standard deviationand. The values less than 0.05 were considered statistically significant.

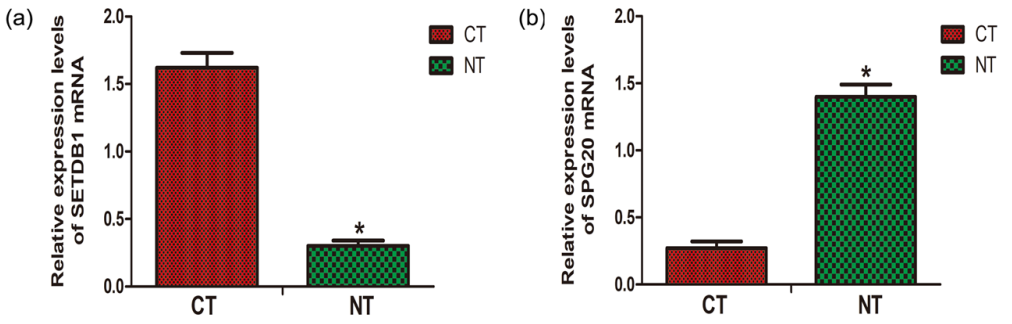

Fig 1. Relative expression of mRNA in tissues. (a) Relative expression of SETDB1 mRNA in cancer and normal tissues.(b)Relative expression of SETDB1 mRNA in cancer and normal tissues.

3. RESULTS

3.1. Relative expression levels of SETDB1 and SPG20 mRNA

As shown in Figure 1(a), when being compared to normal lung tissue mRNA, SETDB1 mRNA relative expression levels of cancer tissues rised significantly. However，the SPG20 mRNA relative expression levels of cancer tissues was lower than the normal lung tissue，as Figure 1(b).

3.2. protein expression levels of SETDB1 and SPG20（Immunohistochemistry）

The results of immunohistochemistry experiments showed that SETDB1 was highly expressed in cancer tissues, with a positive expression rate of 81.67%, higher than 26.67% in normal lung tissue. SPG20 showed a low positive expression rate of 28.33%, lower than 61.67% in normal lung tissue. The difference was statistically significant.(P<0.05). Tab.1, Fig.2

Table 1. Expression levels of SETDB1 and SPG20 in cancerous and normal lung tissues（*n, %*）

Group	n	SETDB1		SPG20	
		Positive	Negative	Positive	Negative
Experimental group	60	49（81.67）	11（18.33）	17（28.33）	43（71.67）
Control group	60	16（26.67）	44（73.33）	37（61.67）	13（21.67）
c2		36.554		22.758	
P		*P*<0.05		*P*<0.05	

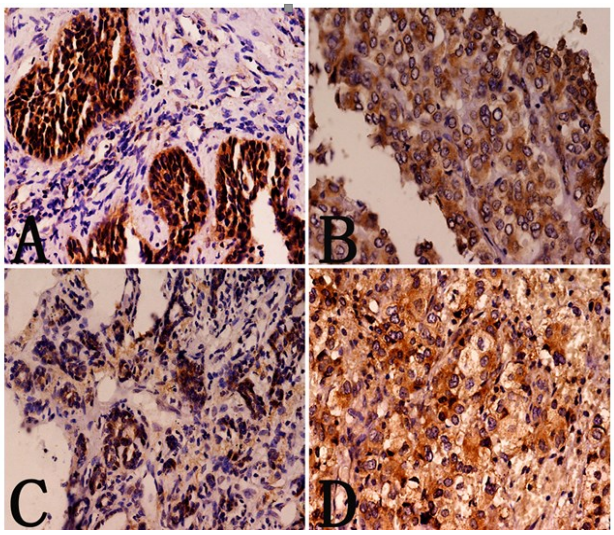

Fig.2 Expression of SETDB1 and SPG20 in cancerous and normal lung tissues (SP-HE ×400) A: SETDB1 is highly expressed in the cancer group; B: SETDB1 has low expression in the normal lung tissue; C: SPG20 has low expression in the cancer group; D: SPG20 shows high expression in the normal lung tissue;

3.3. protein expression levels of SETDB1 and SPG20（Western blot）

To further validate the protein expression levels, we performed protein quantification experiments (Western blot).The results showed that the expression of SETDB1 was (84.16±3.41)% in cancer tissues, higher than (14.76±2.37)% in normal tissue(shown in Figure 3(a, b)). However, expression of SPG20 was (21.37±2.04)% in cancer tissues, lower than (62.64±2.41)% in normal tissue(shown in Figure 3(c, d)).

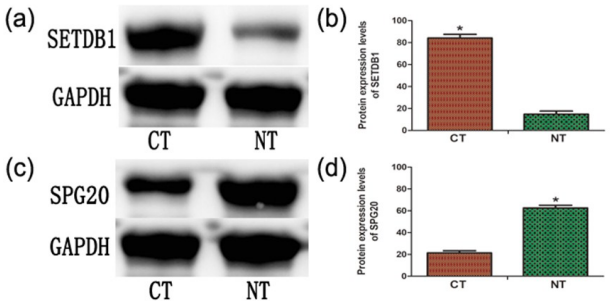

Fig. 3 Protein expression in cancerous and normal lung tissues (CT: Cancerous tissue NT: normal lung tissue) (a, b): SETDB1protein expression (c,d): SPG20protein expression

3.4. SPG20 methylation levels

The result of SPGA20 methylation level in tissues by pyrosequencing showed that the methylation rate of SPG20 gene was (64.75±4.21%) in cancer tissues, which was significantly higher than that (15.36±2.57%) in normal lung tissue.The difference was statistically significant. (P<0.05) Fig.4

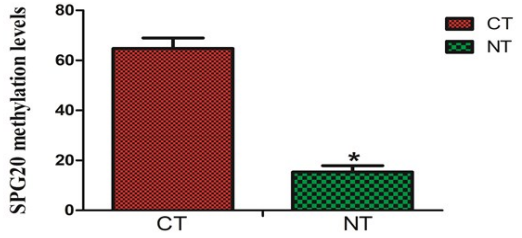

Fig.4 SPG20 methylation levels in lung adenocarcinoma and normal lung tissues(CT: Cancerous tissue NT: normal lung tissue)

3.5. Correlation of SETDB1 and SPG20 methylation

Statistical analysis showed that SETDB1 and SPG20 methylation were correlated in cancer tissues, and the correlation coefficient r=0.642 (P<0.05).（Tab.2）

Table.2 Correlation between SETDB1 and SPG20 methylation in cancerous tissues (n)

SETDB1	SPG20 Methylation		c2	P	r
	Positive	Negative			
Positive	36	13	8.427	0.003	0.642
Negative	3	8			

3.6. Correlation between SETDB1, SPG20 methylation and clinical case characteristics

Positive SETDB1 expression and SPG20 methylation were closely correlated with TNM stage, tissue differentiation, and lymph node metastasis (P<0.05), and not correlation with sex, age, and smoking(P>0.05). (Ta.3). Therefore, these results demonstrate that SETDB1 positive expression and SPG20 methylation are closely related to the malignancy degree and metastasis of lung adenocarcinoma.

3.7. Expression of SETDB1 and SPG20 in cells

The Western blot results showed that SETDB1 was highly expressed in A549 cells. However, the SPG20 showed a low expression level.SETDB1 expression was lower in normal bronchial epithelial cells, but SPG20 level showed a high expression(Fig.5).The expression levels of both proteins in the cell line were consistent with those expressed in the tissues.

Table. 3 Correlation of SETDB1，SPG20 methylation with the clinical case characteristics (n)

Item	Number	SETDB1 positive rate	c^2	P	SPG20 Methylation positive	c^2	P
Sex							
Male	16	13（81.25）	0.003	0.953	5（31.25）	0.050	0.823
Female	44	36（81.82）			12（27.27）		
Age							
≥60	22	17（77.27）	0.046	0.830	7（31.82）	0.115	0.735
< 60	38	32（84.21）			10（26.32）		
TNM stages							
I stage	26	15（57.69）*	8.146	0.013	3（20.00）*	6.124	0.037
II stage	16	14（87.50）**			5（35.71）**		
III stage	18	18（100.00）**			9（50.00）**		
Histodifferentiation							
Well-differentiated	14	9（64.29）*	8.357	0.038	1（7.14）*	9.648	0.008
Moderate differentiated	38	32（84.21）**			12（31.58）**		
Poorly differentiated	8	8（100.00）**			4（50.00）**		
Smoking or not							
Yes	17	13（76.47）	0.044	0.834	3（17.65）	0.787	0.375
No	43	36（83.72）			14（32.56）		
Lymphatic metastasis							
Have	16	16（100.00）*	7.378	0.024	11（68.75）*	8.418	0.004
None	44	33（75.00）**			6（13.64）**		

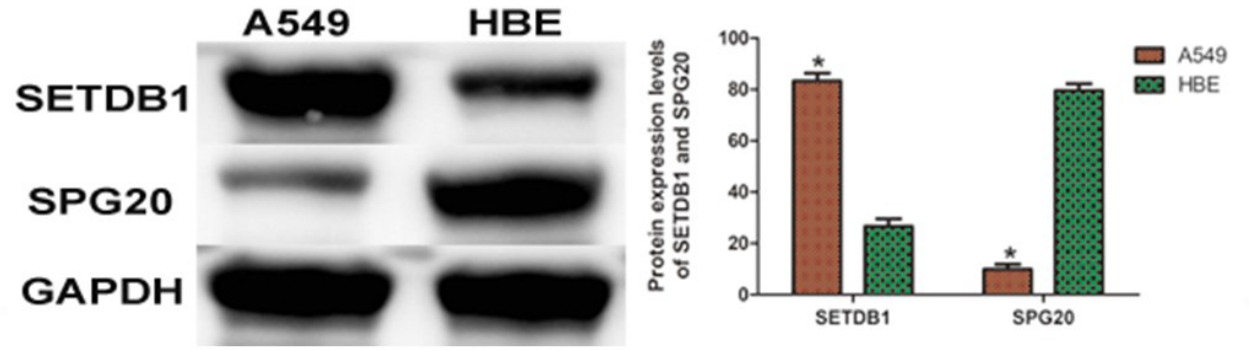

Fig.5 The protein expression of SETDB1 and SPG20 protein in human lung adenocarcinoma A549 cells and normal bronchial cells

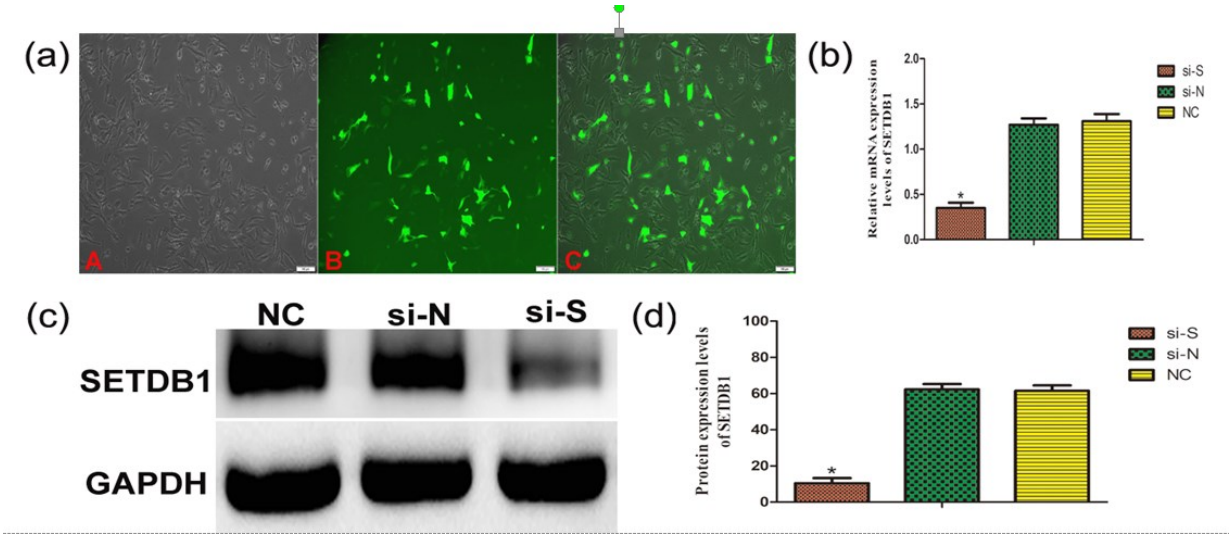

Fig.6 The transfection effect of RNA interferes with the SETDB1（a）:Fluorescence microscopy results of RNA interference SETDB1 transfected A549 cells(A: bright field B: fluorescence microscope C: superposition of A and B); (b):Relative SETDB1 mRNA expression after RNA interference with SETDB1 A549 transfection cells (si-S: SETDB si-R N A group si-N: negative control group NC: blank control group); (c,d):SETDB1 protein expression after RNA interference with SETDB1 A549 transfection cells.

3.8. Result of si-RNA interference SETDB1

The synthetic si-RNA-SETDB1 contained green fluorescence-labeled tags，it had green fluorescence expression after successfully transfected A549 cells (Fig.6a). The SETDB1 relative mRNA expression levels of the cells in group 3 were determined by RT-PCR.The mRNA expression level in the si-S group was significantly lower than that in the si-N group and the NC group(P<0.05, Fig.6b). The Western blot test results showed that theprotein expression of SETDB1 in si-S group was significantly lower than that in si-N group and NC group, which was significantly significant (P<0.05, Fig.6c,d). This indicates that the RNA sequence of SETDB1 is effective.

3.9. Effect of SPG20 methylation and protein expression after RNA interference SETDB1

The result of Pyrosequencing showed that the SPG20 methylation level decreased significantly in si-S group, however, SPG20 methylation levels was no difference between the si-N and NC groups(P<0.05, Tab.4). The results of Western blot showed that the SPG20 protein expression in si-S group was higher than in si-N and NC group after RNA interference downregulated SETDB1.(P<0.05, Fig.7)

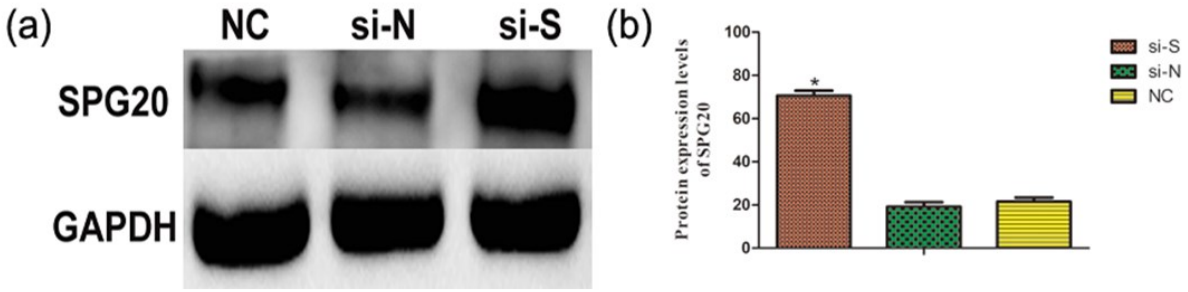

Fig.7 SPG20 protein expression after RNA interference with SETDB1 A549 transfection cells (si-S: SETDB si-R N A group si-N: negative control group NC: blank control group)

Table. 4 The SPG20 methylation rate in each experimental group cells after interference with SETDB1 (1%)

Group	SPG20 Methylation rate
si-S	18.67±0.31*
si-N	74.24±1.35
NC	72.56±1.76

4. DISCUSSION

Early lung adenocarcinoma is mostly asymptomatic, and some patients are advanced at the time of diagnosis, leading to their overall poor prognosis of [10,11].Some progress has been made in the study of gene-targeted therapy for lung adenocarcinoma, but the improvement of its prognosis is still not optimistic.Therefore, improving the early diagnosis rate of lung adenocarcinoma, finding new indicators for evaluating prognosis, and new targets for antitumor therapy are crucial for improving patient prognosis in [12,13].

It have shown that the SPG20 gene encodes Spartin multifunctional protein and is involved in the development of various tumors in humans. The aberrant role of SPG20 in the cell division cycle is associated with tumorigenesis.Studies have confirmed that SPG20 presents a low expression state in gastric cancer, which may be associated with the inhibition of the hypermethylation status in the promoter region of SPG20 in gastric cancer tissues[14].SPG20 is poorly expressed in colorectal cancer and HCC, and its hypermethylation-modification status has been demonstrated as an early event[15,16].According to the previous immunohistochemical experiments, the research group confirmed that SPG20 showed a low expression status in lung adenocarcinoma tissues, and is closely related to the pathological stage, differentiation and lymph node metastasis of lung adenocarcinoma, which is consistent with the low expression status of SPG20 in gastric cancer, rectal cancer and liver cancer [14-17].In this study, the lung adenocarcinoma tissue specimens were detected by immunohistochemistry and protein immunoblotting experiments, and further confirmed that SPG20 protein showed a low expression status in cancer tissues, whereas normal lung tissue showed high expression with significant differences. Analysis of SPG20 methylation in lung adenocarcinoma tissues showed that the SPG20 gene was methylated in lung cancer tissues, and its methylation rate was significantly higher than that in normal lung tissues.The hypermethylation status of SPG20 in lung adenocarcinoma tissues was correlated with the tumor TNM stage, differentiation, as well as the metastasis of lymph nodes, indicating that the hypermethylation of the SPG20 gene was correlated with the proliferation, invasion, and metastasis of lung adenocarcinoma. The positive expression of SPG20 gradually decreased with the increased TNM stage and cancer tissue differentiation of lung adenocarcinoma, and the positive expression with lymph node metastasis decreased significantly. This indicates that SPG20 may act as a “tumor suppressor gene” in the development of lung adenocarcinoma, and the low expression state of SPG20 attenuates its tumor suppressor effect, which then promotes the proliferation, invasion and metastasis of lung adenocarcinoma.

Methylation modification requires the involvement of methyltransferases. The dynamics of methyltransfer-

ases make the DNA hypermethylation and hypomethylation in a mutually irreversible dynamics, therefore, the role of methyltransferase in the methylation modification process is crucial[18].

It was shown that the abnormal expression of methyltransferases was found to be associated with their abnormal methylation, and it is involved in the proliferation, invasion, and metastasis of various tumors [19-22].The SET domain clade-type 1 (SETDB1) is a member of the histone methyltransferases family, displaying multiple functional in biological networks involved in transcriptional repression and autosomal gene silencing[23].SETDB1 has a variety of biological functions including regulating the growth and proliferation of embryonic stem cells and regulating mouse cartilage development[24].Recent studies have found that SETDB1 is upregulated in various human cancers, such as colon cancer [25], liver cancer [26], gastric cancer [27], lung cancer [28], breast cancer [29], etc., which is closely related to the degree of malignancy and metastasis of tumors.Xiao[30] et al found that SETDB1 was amplified and highly expressed in breast cancer tissues, and that silencing SETDB1 inhibited the formation of adherent-dependent foci in breast cancer cells. This suggests that SETDB1 may be one of the potential oncogenic drivers in breast cancer, and that its high expression state promotes breast cancer initiation and development. Na[30] et al found that low expression of SETDB1 inhibited tumor growth in tumor cells and nude mouse models, while its overexpression increased tumor aggressiveness, and SETDB1 overexpression was associated with enhanced sensitivity to tinomycin-mediated growth inhibition.In this study, SETDB1 levels were measured in lung adenocarcinoma by immunohistochemistry and protein immunoblotting, which showed positive SETDB1 expression in cancer tissues and was significantly different from normal lung tissues.The statistical analysis results are shown that the expression level of SETDB1 was closely related to the TNM stage, tissue differentiation degree and lymph node metastasis of lung adenocarcinoma; The positive expression of SETDB1 increased with the increase of TNM stage and cancer tissue differentiation of lung adenocarcinoma, and the positive expression of lymph node metastasis was increased significantly, which obviously played the role of "promoting cancer".This is consistent with foreign studies, which the high expression status of SETDB1 in NSCLC lung cancer tissues, which has a strong correlation with lung cancer [30].

The research group's preliminary research has found that the low expression of SPG20 in lung adenocarcinoma tissues is closely related to its methylation in [17].Studies have confirmed that SETDB1, acting as a methyltransferase, can catalyze the methylation of various genes, inhibit the expression of genes, and then regulate tumor genesis and development.Whether SETDB1 acts as a methyltransferase is associated with the methylation modification of SPG20 in lung adenocarcinoma, and the correlation and interaction mechanism of the two in lung adenocarcinoma have been rarely studied.The results of the correlation analysis of SPG20 methylation and SETDB1 in cancer tissues showed that high SETDB1 expression and high levels of SPG20 methylation in lung adenocarcinoma tissues, both were positively correlated.Expression of SETDB1 and SPG20 protein was further examined in lung adenocarcinoma cell lines, the results confirm that SETDB1 in lung adenocarcinoma cell lines was highly expressed in lung adenocarcinoma cells and SPG20; SETDB1 expression was low in normal bronchial epithelial cells and high in SPG20, which is consistent with the results tested in lung adenocarcinoma tissues.

The correlation of methyltransferase SETDB1 with SPG20 methylation was further verified by in vitro cell assays, result display that the methylation rate of the SPG20 gene was significantly decreased after SETDB1 knockdown by RNA interference,SPG20 protein expression increased. Combining the correlation results of SPG20 methylation and SETDB1, the author speculated that SETDB1 acting as a methyltransferase in lung adenocarcinoma promotes SPG20 methylation, which subsequently inhibited the expression of SPG20, increasing the malignancy of lung adenocarcinoma. However, the specific mechanism of action in the development of lung adenocarcinoma still needs to be further explored. In the future re-

search, SETDB1 and SPG20 methylation can be taken as the entry point to deeply study the specific carcinogenesis mechanisms, so as to provide a new theoretical basis for the gene-targeted therapy of lung adenocarcinoma.

In conclusion, SPG20 methylation and SETDB1 are closely related to the stage, differentiation and metastasis of lung adenocarcinoma, and they show a positive correlation. They may work together to promote the development of lung adenocarcinoma, and have potential applications in the diagnosis and treatment of lung adenocarcinoma.

Supplementary Information

Acknowledgements

Not applicable.

Author contributions

BS Zhao and ZY Liang conceived the hypothesis and designed the experiments. BS Zhao, JT Huang, ZY Liang performed the experiments. JT Huang and ZY Liang analyzed the data, visualized the fgures and drafted the manuscript. BS Zhao revised the article.

All authors read and approved the fnal manuscript.

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Availability of data and materials

The data included in this investigation are available from the corresponding author.

Declarations

Ethics approval and consent to participate

All experimental protocols were implemented following the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the Ethics Committee of Affiliated Hospital of Chengde Medical University

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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