doi: 10.4149/gpb_2025005

The expression of circSATB2, PEAK1 in non-small cell lung cancer tissue and their relationships with clinical pathological characteristics, as well as postoperative recurrence and metastasis

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Abstract. We studied the circSATB2 and PEAK1 expression in NSCLC tissues and their associations with clinicopathological features, recurrence, and metastasis. Tumor and adjacent tissues from 98 NSCLC patients (2019–2020) were analyzed using qPCR (circSATB2) and immunohistochemistry (PEAK1). Pearson correlation assessed circSATB2-PEAK1 mRNA relationships. Survival analysis compared high/low expression groups. Cox regression identified risk factors for recurrence/metastasis. circSATB2 and PEAK1 mRNA were upregulated in NSCLC tissues, correlating with lymph node metastasis and advanced TNM stage (IIIa). circSATB2 positively correlated with PEAK1 mRNA. High expression of both predicted poorer survival (Kaplan-Meier). Multivariate analysis identified high circSATB2, high PEAK1, lymph node metastasis, and stage IIIA as independent risk factors for postoperative recurrence/metastasis. In conclusion, elevated circSATB2 and PEAK1 in NSCLC are linked to aggressive clinicopathological features and poor prognosis, serving as biomarkers for recurrence/metastasis risk.

Key words: Non-small cell lung cancer — circSATB2 — PEAK1 — Clinical pathological characteristics — Recurrent metastasis

Introduction

Lung cancer is the main cause of cancer-related deaths in the world, with 1.59 million deaths every year (Bade and Dela Cruz 2020). Non-small cell lung cancer (NSCLC) is the main pathological type of lung cancer, and its incidence has been rising in recent years, accounting for about 85% of all cases. At present, surgery, radiotherapy and chemotherapy and targeted therapy are the main clinical treatments. Despite the progress and improvement in surgery and medical treatment, the 5-year survival rate of metastatic NSCLC is only 5%. Therefore, there is an urgent need for new sensitive biomarkers for early detection of NSCLC and target molecules for developing new therapies (Chang et al. 2023).

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Circular RNA (circRNA) has received extensive attention in recent years. circRNA is a special kind of non-coding RNA, which forms a closed-loop structure and is not easily degraded by RNase in cells, so it is relatively stable. circRNA plays a role in many physiological and pathological processes, including the occurrence and development of tumors. In particular, circSATB2, a newly discovered circRNA, has gradually attracted researchers' attention for its role in non-small cell lung cancer. circSATB2 is located on human chromosome 2q33.1, which not only plays a role in the development of brain tissue, but also involves the proliferation and migration of vascular smooth muscle cells, showing that it has multiple functions in cell biology (Paudel et al. 2020; Pu et al. 2021). In addition, as a "molecular sponge" of microRNA, circSATB2 can interfere with the normal function of microRNA, and then affect the gene expression and cell signal transmission path that microRNA can regulate. This mechanism plays a key role in the proliferation, apoptosis, invasion, autophagy and sensitivity regulation of tumor cells to radiotherapy and chemotherapy (Zhang et al. 2021).

PEAK1 (Pseudopodium-enriched atypical kinase 1) belongs to the NKF3 family, and its coding gene is located at 15q24.3. As an atypical tyrosine kinase, it plays a regulatory role in cytoskeleton recombination, migration and invasion, and is closely related to tumor development and metastasis (Zuidema et al. 2022). Studies have shown that the abnormal expression of PEAK1 in pancreatic cancer can activate the signal transduction of Yes-related protein signaling pathway, up-regulate the expression of oncogenes such as c-Myc, and promote the malignant proliferation and invasion of tumor cells (Zuidema et al. 2017). Studies have shown, that circSATB2 in exosomes, as a molecular sponge of microRNA-330-5p, upregulates the expression of PEAK1 in lung cancer cells and promotes the malignant development of lung cancer (Abdel-Rahman 2018). However, the expression and significance of circSATB2 and PEAK1 in NSCLC are still unclear. Therefore, it is very important to actively explore the expression of circSATB2 and PEAK1 in non-small cell lung cancer and their relationship with clinicopathological features, as well as their potential impact on postoperative recurrence and metastasis, in order to provide new ideas and targets for the treatment of NSCLC.

Materials and Methods

Research object

From January 2019 to January 2020, 98 patients with NSCLC in our hospital were selected, and the cancer and adjacent tissues (more than 2 cm from the edge of the cancer tissue) obtained during the operation were taken. After that, all tissue samples were immediately stored in liquid nitrogen, and then transferred to the refrigerator for long-term storage for subsequent RNA extraction. This study was approved by the hospital ethics committee (ethics batch number: JCRGYY-EC-KY-2018-04).

Inclusion criteria: 1) All patients with NSCLC underwent lobectomy, mediastinal lymph node sampling or systematic lymph node dissection, all margins were negative, and no lesions remained during the operation, and NSCLC was diagnosed by postoperative histopathology; 2) The 8th edition of union for international cancer control lung cancer staging is for patients with stage I, II and III, who are evaluated by surgeons as operable; 3) The expected postoperative survival time is more than 3 months; 4) Patients and their families have signed the informed consent of this study; 5) General clinical data and imaging data are complete.

Exclusion criteria: 1) Receiving neoadjuvant chemotherapy or targeted therapy before operation; 2) Combined with other malignant tumors; 3) Complicated with dysfunction of heart, liver, kidney and other organs; 4) Death due to perioperative complications. 57 males and 41 females; the age ranged from 31 to 85 years, with an average of (64.22 ± 7.23) years. Pathological types: 60 cases of lung adenocarcinoma and 38 cases of lung squamous cell carcinoma; Tumor diameter: <3 cm in 57 cases and ≥3 cm in 41 cases; TNM staging of tumor: 62 cases in stage I~II, 36 cases in stage IIIA; there were 59 cases with high differentiation degree and 39 cases with low differentiation degree. 34 cases were accompanied by lymph node metastasis.

Real-time fluorescence quantitative PCR detection

The cancer and adjacent tissues were added into RIPA lysate, ground in liquid nitrogen, centrifuged at 3000 rpm/min, and the total RNA in the tissues was extracted with Trizol reagent. After reverse transcription of total RNA into cDNA, fluorescence quantitative PCR was performed. Primers were designed and synthesized by Beijing Tian Ikki Yuan Biology Company. circSATB2, F: 5'-GCAGTTGGACGGCTCTCTT-3', R: 5'- CACCTTCCCAGCTTGATTATTCC-3'; PEAK1, F: 5'- TGGAGATTCGCATGAGAGGG-3', R: 5'- TCCTTGTCCATAAGAGACCACA-3'; the expression of GAPDH, F: 5'-GACAGTGGCCGACATGCTAC-3', R: 5'- AGGCAAGTCTTCCAACTTTGAA-3'. The total system is 10 μl: template cDNA 0.5 μl, 2×SYBR Green Premix 5 μl, upstream and downstream primers are 0.5 μl, and DEPC water is 3.5 μl. Reaction procedure: pre-denaturation at 95°C for 5 min, denaturation at 95°C for 30 s, annealing at 60°C for 34 s, and extension at 72°C for 30 s, a total of 35 cycles. $2^{-\Delta\Delta Ct}$ method was used to analyze the relative expression of circSATB2 and PEAK1 mRNA in tissues.

Immunohistochemistry

The cancer and adjacent tissues were fixed in fixed solution for 16 h, sliced in paraffin, baked in a constant temperature box at 60°C for 20 min, dewaxed with xylene for 10 min, hydrated with gradient ethanol, incubated with 3% hydrogen peroxide at room temperature for 20 min, in 0.01 M citrate solution at 100°C for 5 min, cooled to room temperature, and sealed with 3% sheep serum for 2 h. After dropping the primary antibody of PEAK1 (1:500, ab121865, Abcam), it was incubated at 4°C for 12 h. The secondary antibody (1:1000, ab205718, Abcam) was incubated at room temperature for 1 h. DAB developed for 5 min, hematoxylin was stained again, 1% hydrochloric acid alcohol was differentiated, gradient ethanol was dehydrated, xylene was re-transparent, and then neutral gum was sealed. Take photos under the microscope (model DX31, Olympus, Japan) and observe the dyeing results. All the pathologists read the films blindly, and observed and counted them under the microscope of 200 times. Each slice randomly selected 5 fields, and each field counted cells. Yellow or brown in cell membrane or cytoplasm was used as positive staining. Positive cell ratio score: no positive cells are recorded as 0; the percentage of

positive cells <30% is 1 point; the percentage of positive cells is 30% ~ 60%, which is 2 points; the percentage of positive cells >60% was recorded as 3 points. Color rendering score: those whose color rendering is similar to the background will be scored as 0; light color, slightly higher than the background, 1 point; moderate color development, significantly higher than the background, scored 2 points; strong dyeing, dark brown color is recorded as 3 points. The product of positive cell ratio score and color rendering score ≥2 is positive, and <2 is negative. In Cox regression analyses, reference categories were coded as 0 and explicitly labeled in Table 2.

Follow-up

Patients with NSCLC were followed up regularly in outpatient clinic, and the first clinical follow-up was conducted one month after operation, once every three months in the first year after operation and once every six months after the second year. Chest CT scan was performed every 6 months. Follow-up covered the patient's survival, postoperative treatment plan and recent review results, follow-up lasted for 3 years until May 2023. The end point of follow-up was tumor recurrence, metastasis or death during follow-up. Progression-free survival events are defined as the time from postoperative start to tumor recurrence, metastasis or death due to tumor progression. This study started from the day of operation until the local or distant recurrence of the tumor was found by imaging examination and confirmed by histopathology. Recurrence is defined as the occurrence of tumor in the following parts: ipsilateral lung, bronchial stump, or regional lymph nodes (subgingival lymph nodes, paraesophageal lymph nodes, ipsilateral or contralateral mediastinal lymph nodes, supraclavicular lymph nodes or hilar lymph nodes). Distant metastasis is defined as including metastasis of contralateral lung, liver, adrenal gland, brain, bone or other parts.

Statistical analysis

SPSS26.0 software was used to analyze the data. The measurement data in accordance with the normal distribution are expressed as mean standard deviation, and two independent samples are used for *t*-test between groups. Counting data were expressed by percentage (%), and chi-square test was used to compare between groups. Pearson correlation analysis was used to analyze the correlation between circSATB2 and PEAK1 mRNA expression in cancer tissues. Kaplan-Meier survival curve and Log-Rank test were used to compare the differences between different circSATB2 and PEAK1 mRNA expression curves. Univariate and multivariate Cox proportional hazard models were used to analyze the influencing factors of postoperative recurrence and metastasis in patients with NSCLC. $p < 0.05$ is statistically significant.

Results

circSATB2 and PEAK1 are up-regulated in NSCLC

The relative expressions of circSATB2 and PEAK1 mRNA in NSCLC were (3.14 ± 0.33) and (2.78 ± 0.29), which were higher than those in the adjacent tissues (1.02 ± 0.23) and (0.84 ± 0.26), and the difference was statistically significant (t = 52.175, $p < 0.001$; t = 49.309, $p < 0.001$). The positive staining of PEAK1 protein in NSCLC cancer tissue was located in cytoplasm and cell membrane. The positive rate of PEAK1 in cancer tissues was 65.31% (62/98), which was higher than that in adjacent tissues (6.12%, 8/98), with statistical significance ($c^2 = 64.800$, $p < 0.001$; Fig. 1). Pearson correlation analysis showed that there was a significant positive correlation between circSATB2 and PEAK1 mRNA expression in NSCLC (r = 0.720, $p < 0.001$).

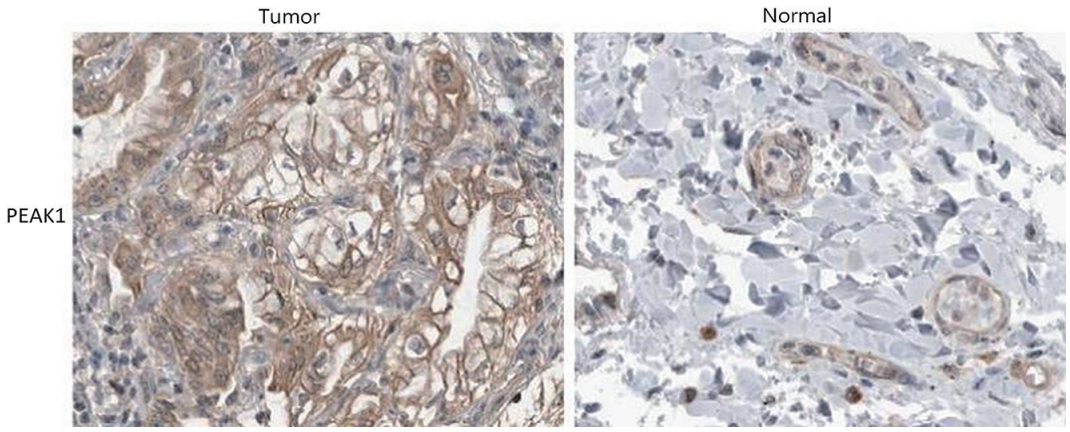

Figure 1. Expression of PEAK1 protein in cancer (**A**) and adjacent (**B**) tissues (immunohistochemistry, 200×).

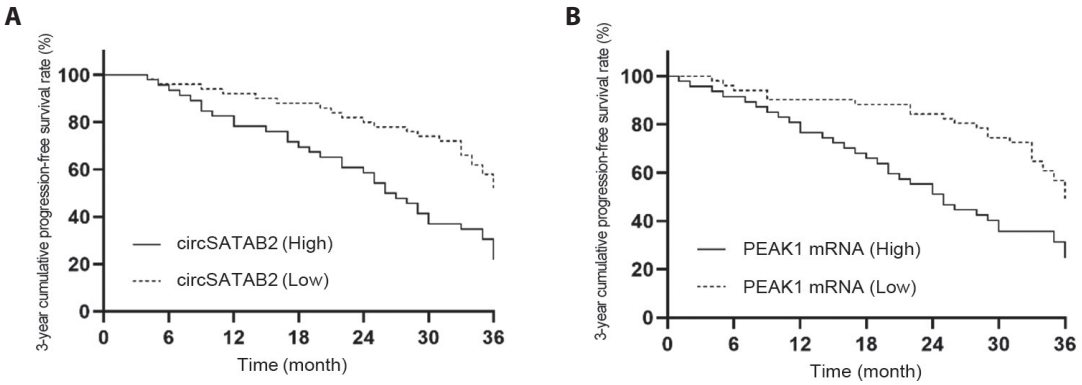

Figure 2. Kaplan-Meier curve analysis of the influence of circSATB2 (**A**) and PEAK1 (**B**) mRNA expression on postoperative recurrence and metastasis of NSCLC patients.

Comparison of circSATB2 and PEAK1 mRNA expression in NSCLC cancer tissues with different clinicopathological features

The expression of circSATB2 and PEAK1 mRNA in NSCLC with lymph node metastasis and TNM stage IIIA was higher than that without lymph node metastasis and TNM stage I~II, and the difference was statistically significant (all $p < 0.05$; Table 1).

Effect of circSATB2 and PEAK1 mRNA expression on postoperative recurrence and metastasis of NSCLC patients

During the follow-up, there were 60 cases with recurrence and metastasis, and the overall 3-year progression-free survival rate was 38.78% (38/98). The 3-year overall progression-free survival rates of high and low expression groups of circSATB2 were 25.00% (12/48) and 52.00% (26/50), re-

Table 1. Comparison of circSATB2 and PEAK1 mRNA expression in NSCLC patients with different clinicopathological features

Parameter	*n*	circSATB2	t	*p*	PEAK1 mRNA	t	*p*
Age			1.336	0.185		1.666	0.099
<60 years old	45	3.09 ± 0.31			2.72 ± 0.25		
≥60 years old	53	3.18 ± 0.35			2.83 ± 0.33		
Gender			1.723	0.088		1.661	0.100
male	57	3.19 ± 0.36			2.82 ± 0.31		
female	41	3.07 ± 0.31			2.72 ± 0.27		
Pathological types			1.552	0.124		1.871	0.064
glandular cancer	60	3.10 ± 0.33			2.74 ± 0.25		
squamous carcinoma	38	3.21 ± 0.36			2.85 ± 0.33		
Degree of differentiation			1.491	0.139		0.814	0.418
high	59	3.10 ± 0.30			2.78 ± 0.32		
low	39	3.20 ± 0.36			2.83 ± 0.26		
Tumor diameter			1.045	0.299		0.854	0.395
<3 cm	57	3.11 ± 0.31			2.76 ± 0.25		
≥3 cm	41	3.18 ± 0.35			2.81 ± 0.33		
Lymph node metastasis			22.332	<0.001*		22.170	<0.001*
yes	34	4.20 ± 0.41			3.65 ± 0.35		
no	64	2.58 ± 0.30			2.32 ± 0.24		
TNM staging			22.280	<0.001*		24.069	<0.001*
I~II	62	2.54 ± 0.32			2.25 ± 0.26		
IIIA	36	4.18 ± 0.40			3.70 ± 0.33		

NSCLC, non-small cell lung cancer; circSATB2, circular RNA SATB2; PEAK1, Pseudopodium-enriched atypical kinase 1; * significantly different ($p < 0.05$).

Table 2. Single factor Cox regression analysis

Factor	Assignment	B	SE	Wald χ^2	*p*	HR	95%CI
Gender	(male = 1, female = 0)	0.379	0.250	2.298	0.261	1.461	0.895~2.385
Age	≥60 years old = 1, <60 years old = 0	0.311	0.247	1.585	0.448	1.365	0.841~2.215
Tumor Diameter	≥3 cm = 1, <3 cm = 0	0.287	0.210	1.868	0.313	1.332	0.883~2.011
Tumor Type	1 = Adenocarcinoma, 0 = Squamous carcinoma	0.303	0.225	1.814	0.339	1.354	0.871~2.104
Lymph Node Metastasis	1 = Yes, 0 = No	0.620	0.182	11.605	<0.001	1.859	1.301~2.656
Differentiation Degree	low differentiation = 1, high/medium differentiation = 0	0.344	0.191	3.244	0.134	1.411	0.970~2.051
TNM Staging	IIIA staging = 1, I~II staging = 0	0.476	0.141	11.397	<0.001	1.610	1.221~2.122
circSATB2	high expression = 1, low expression = 0	0.607	0.205	8.767	<0.001	1.835	1.228~2.742
PEAK1 mRNA	high expression = 1, low expression = 0	0.556	0.172	10.449	<0.001	1.744	1.245~2.443

SE, standard error; Wald χ^2, Wald Chi-square test statistic; HR, hazard ratio; CI, 95% confidence interval.

spectively. The overall 3-year progression-free survival rates of high and low expression groups of PEAK1 mRNA were 27.66% (13/47) and 49.02% (25/51), respectively. Kaplan-Meier curve analysis showed that the 3-year cumulative progression-free survival rate of NSCLC patients with high expression of circSATB2 and PEAK1 mRNA was lower than that of patients with low expression of circSATB2 and PEAK1 mRNA, respectively, and the difference was statistically significant (Log-Rank χ^2=11.270, p = 0.001; Log-Rank χ^2 = 10.010, p = 0.002; Fig. 2).

Univariate and multivariate Cox regression analysis

With the recurrence and metastasis of NSCLC patients during the follow-up period as the dependent variable (1 = Yes, 0 = No), univariate and multivariate Cox regression analysis found that high expression of circSATB2, high expression of PEAK1 mRNA, lymph node metastasis and TNM stage IIIA were independent risk factors for postoperative recurrence and metastasis of NSCLC patients (Table 2 and 3).

Discussion

NSCLC is a malignant tumor originating from bronchial mucosa, and its morbidity and mortality rank first in China. In recent years, with the improvement of people's living standards and the enhancement of health awareness of regular physical examination, the early detection rate and operation rate of lung cancer have been improved, but postoperative recurrence and metastasis are still important reasons for the high mortality rate of NSCLC patients (Dezube and Jaklitsch 2020). TNM staging is an important factor in clinical follow-up and prognosis evaluation of NSCLC patients, but about 25% ~ 50% patients still have tumor recurrence or metastasis even after extensive tumor resection. Therefore, more sensitive prognostic biomarkers are urgently needed for risk stratification and better evaluation of tumor recurrence and metastasis.

Circular RNA is a noncoding RNA with 5'–3' phosphodiester bond, which has high stability and is widely involved in the pathophysiological processes of eukaryotic differentiation, development, inflammation and immunity (Chen et al. 2021). circSATB2 is a kind of circular RNA discovered in recent years, which can be used as a molecular sponge to bind microRNA, regulate the expression of matrix attachment region's DNA binding protein, and participate in the process of transcription regulation and chromatin remodeling (Dell'Orco et al. 2020). Studies have shown that the expression level of circSATB2 in circulating serum exosomes of unresectable stage III NSCLC patients is increased, which is closely related to the sensitivity and survival prognosis of concurrent radiotherapy and chemotherapy, and is a potential tumor marker for NSCLC. In this study, the up-regulation of circSATB2 expression in NSCLC tissues is related to lymph node metastasis and TNM staging, suggesting that circSATB2 is involved in the occurrence and development of NSCLC diseases. The increased expression of circSATB2 in NSCLC is related to the abnormal cyclization of mRNA after transcription of SATB2 gene. Studies have shown that the RNA binding protein MBL can recognize the BHB motif in the intron of SATB2 gene, promote the tRNA splicing endonuclease complex to cut the intron of SATB2 precursor mRNA, and lead to the cyclization of the end connection of the intron to form circSATB2 (Zhang et al. 2021). circSATB2 can be used as a molecular sponge to bind microRNA-326, up-regulate the expression of Fascin homologue 1 and Actin binding protein 1 in tumor cells, and promote the proliferation, migration and invasion of normal human bronchial epithelial cells and NSCLC tumor cells (Zhang N et al. 2020). In this study, the progression-free survival prognosis of NSCLC patients with high expression of circSATB2 is poor; suggesting that detecting the expression of circSATB2 in NSCLC cancer tissue is helpful to evaluate the postoperative recurrence and metastasis of patients. By

Table 3. Multivariate Cox regression analysis

Factor	B	SE	Wald χ^2	*p*	HR	95%CI
Lymph Node Metastasis	0.535	0.162	10.906	<0.001	1.707	1.243~2.346
TNM Staging	0.462	0.150	9.486	<0.001	1.587	1.183~2.130
circSATB2	0.517	0.185	7.810	<0.001	1.677	1.167~2.410
PEAK1 mRNA	0.503	0.169	8.859	<0.001	1.654	1.187~2.303

SE, standard error; Wald χ^2, Wald Chi-square test statistic; HR, hazard ratio; CI, 95% confidence interval.

analyzing its mechanism, the expression of circSATB2 in NSCLC can be transmitted to adjacent tumor cells through exosomes in the form of paracrine, which can inhibit the expression of microRNA-330-5p and tumor inhibition effect, up-regulate the expression of PEAK1, promote the malignant proliferation and metastasis of lung cancer tumor cells, and increase the risk of postoperative recurrence and metastasis of patients (Zhu et al. 2022). Other studies have shown that the overexpression of circSATB2 can reduce the sensitivity and short-term therapeutic effect of synchronous radiotherapy and chemotherapy in NSCLC patients, and promote the recurrence and metastasis of NSCLC tumors.

PEAK1 belongs to 3 members of the new kinase family. As a non-receptor protein kinase, PEAK1 is widely expressed in many tissues and organs of human body, and participates in cell proliferation, differentiation, migration and invasion. Studies have shown that PEAK1 can bind with many downstream signal molecules containing SH2, such as SRC and CRK, and activate cell mitosis, cell migration and other signal pathways (Zuidema et al. 2021). Studies have shown that the up-regulation of PEAK1 expression in melanoma can activate Janus-related kinase/transcription signal transduction and activator 3 pathway, and play a biological role in promoting tumor growth, invasion and metastasis (Pan et al. 2021). The expression of PEAK1 mRNA and protein in NSCLC is obviously up-regulated, which is related to lymph node metastasis and TNM staging, suggesting that PEAK1 is involved in the tumor progression of NSCLC. Up-regulation of PEAK1 expression in NSCLC is regulated by transcription of cyclin 5-like protein. Studies have shown that the expression of cyclin 5-like protein in NSCLC is obviously up-regulated, which can directly combine with the PEAK1 promoter and promote the transcription of PEAK1, activate the extracellular signal-regulated kinase 1/2 signaling pathway, and promote the malignant proliferation and invasion of tumor cells (Zhang C et al. 2020). Another scholar reported that the overexpression of PEAK1 in lung cancer can activate Janus-related kinase 2 signaling pathway, promote epithelial-mesenchymal transition of tumor cells, up-regulate the expression of matrix metalloproteinases 2 and 9, and enhance the invasion and metastasis of tumor cells (Ding et al. 2018). In this study, the prognosis of NSCLC patients with high expression of PEAK1 mRNA is poor; suggesting that detecting the expression of PEAK1 mRNA in NSCLC cancer tissue is helpful to evaluate the postoperative recurrence and metastasis of NSCLC patients. By analyzing the reasons, the overexpression of PEAK1 in NSCLC can not only promote the proliferation, invasion and migration of tumor cells, but also reduce the sensitivity of tumor cells to adriamycin therapy, resulting in some tumor cells remaining during postoperative chemotherapy, resulting in postoperative recurrence and metastasis. By inhibiting the expression of PEAK1, the chemotherapy resistance of tumor cells can be reversed, the sensitivity of tumor to chemotherapy treatment can be enhanced, and the risk of tumor recurrence and metastasis can be reduced (Wang et al. 2022). In this study, there was a significant positive correlation between circSATB2 and PEAK1 mRNA expression in NSCLC, suggesting that both of them play a synergistic biological role in promoting cancer in NSCLC. Studies have confirmed that circSATB2 in NSCLC can bind microRNA-330-5p as a molecular sponge, thus enhancing the stability of the downstream target gene PEAK1 mRNA of microRNA-330-5p and promoting the protein expression of PEAK1 and the malignant progress of tumor. However, whether it can be used in the treatment of NSCLC by intervening of the interaction between them remains to be further studied in the future.

Conclusion

To sum up, the expressions of circSATB2 and PEAK1 are increased in NSCLC tissues, which are related to lymph node metastasis and TNM staging of NSCLC patients, and both of them play an important role in tumor promotion in NSCLC. High expression of circSATB2, high expression of PEAK1 mRNA, lymph node metastasis and TNM stage IIIA are independent risk factors for postoperative recurrence and metastasis of NSCLC patients. Detecting the expression of circSATB2 and PEAK1 in NSCLC cancer tissues is helpful for clinicians to evaluate the risk of postoperative recurrence and metastasis of NSCLC patients. However, there are still some shortcomings in this study. The sample size of this study is limited, and there may be some bias in the inclusion of the research objects, which needs to be further studied by designing large-sample multi-center clinical trials in the future.

Ethics approval and consent to participate. This study was approved by the ethics committee of Shenzhen Jingcheng Healthcare Group Rugao Hospital (ethics batch number: JCRGYY-EC-KY-2018-04). All subjects signed the informed consent. All methods were carried out in accordance with relevant guidelines and regulations.

Availability of data and materials. The datasets used during the present study are available from the corresponding author upon reasonable request.

Conflict of interest. All authors declare that they have no competing interest.

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Received: July 17, 2024
Final version accepted: February 13, 2025