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Original Article

Expression of RSK4 protein in non-small cell lung cancer tissues, adjacent tissues and its correlation with clinicopathological features

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Abstract: Objective: To evaluate the expression of Ribosomal S6 kinase 4 (RSK4) protein in non-small cell lung cancer (NSCLC) tissues and adjacent non-tumor tissues, and to elucidate its correlation with clinicopathological features of NSCLC. Methods: We analyzed 100 NSCLC patients treated at the Second Affiliated Hospital of Zhejiang University School of Medicine from June 2020 to June 2022. Patient demographics and clinical data, including gender, age, history of diabetes, tumor location, degree of tumor differentiation, lymph node metastasis, and clinical stage, were collected. RSK4 protein expression was assessed in tissue samples via immunohistochemical staining. Results: RSK4 protein was positively expressed in 35.00% of cancerous tissues, significantly lower than the 69.00% observed in adjacent non-tumor tissues ($P < 0.05$). Patients with lower tumor differentiation, advanced Tumor Node Metastasis (TNM) stages, and lymph node metastases showed significantly reduced RSK4 expression compared to those with higher differentiation, earlier TNM stages, and no lymph node metastases (all $P < 0.05$). Cox regression analysis indicated that TNM stage, low differentiation, and lymph node metastases significantly influenced RSK4 expression (all $P < 0.05$). Survival analysis revealed a higher positive prognosis survival rate of 74.29% (26/35) among patients with positive RSK4 expression, versus 53.85% (35/65) in those with negative expression ($P < 0.05$). Spearman correlation analysis demonstrated a significant positive correlation of RSK4 expression with TNM stage, lymph node metastasis, and patient prognosis (all $P < 0.05$). Conclusion: Positive RSK4 expression in NSCLC tissues is significantly correlated with advanced cancer stage, poor differentiation, and presence of lymph node metastasis, suggesting a potential tumor suppressor role for RSK4 in NSCLC. This association underscores its prognostic relevance in NSCLC patients.

Keywords: RSK4 protein, non-small cell lung cancer, expression, clinicopathology, correlation, prognosis

Introduction

Lung cancer is one of the most prevalent and lethal malignant tumors worldwide [1]. It leads both in incidence and mortality among all cancers. Non-small cell lung cancer (NSCLC) is the predominant pathological type, constituting approximately 82.5% of lung cancer cases [2, 3]. Patients with advanced NSCLC often present without specific symptoms, resulting in exceedingly low survival rates at advanced stages [4]. NSCLC is frequently treated clinically, though treatments are often associated with significant complications [5]. Ribosomal S6 kinase 4 (RSK4) is a member of the ribosomal S6 kinase (RSK) family, known for its extensive regulatory roles in cellular functions including protein synthesis, cell proliferation, and apoptosis [6]. Abnormal expression of RSK4 has been documented in breast cancer cell lines and tumors, suggesting its involvement in oncogenesis [7]. RSK4 is unlike other family members which are expressed variably in embryonic and adult tissues and have different cellular roles [8]. In somatic cells, RSK stimulates cell division through phosphorylation of P27Kip18 and glycogen synthetic kinase-39-11. RSK4 promotes protein synthesis and cell

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growth through phosphorylation substrates such as extension factor-2 kinase L2, glycogen synthetic kinase-3, transcription initiation factor, and tuberS-2 protein; it affects apoptosis by acting on Bad, and it affects transcription by c-fox6 and estrogen receptor. Some studies have found that the RSK4's expression is notably diminished in malignant tumors like lung and breast cancer, aligning with its potential role as a tumor suppressor [9]. This study expands on previous research by examining RSK4 expression in a larger cohort of NSCLC cases using immunohistochemistry (IHC) to explore its pathological relationship with NSCLC. Key findings are summarized below.

Materials and methods

General information

A retrospective analysis was conducted on a cohort of 100 patients diagnosed with advanced NSCLC treated at the Second Affiliated Hospital of Zhejiang University School of Medicine from June 2020 to June 2022. The sample included 56 males and 44 females, aged between 45 and 76 years. All patients met the diagnostic criteria for advanced NSCLC and were histologically confirmed as either adenocarcinoma or squamous carcinoma. Inclusion criteria required patient survival for more than three months post-diagnosis. Patients were excluded if they had active infections, central nervous system metastasis; severe pain unrelated to lung cancer metastases, severe mental illness, had declined treatment, or were unable to complete data collection due to any reason. Patients who received other anti-tumor treatments such as systemic chemotherapy or local radiotherapy during the study period were also excluded. This study received approval from the Hospital Medical Ethics Committee.

Research methods

Data collection: Demographic and clinical data collected included sex, age, history of diabetes, tumor location, degree of tumor differentiation, presence of lymph node metastasis, and clinical stage. These variables served as indicators to explore the relationship between RSK4 protein expression and clinical outcomes in NSCLC patients.

Immunohistochemistry: All patients underwent surgical resection to obtain carcinoma and adjacent tissues, with the latter defined as tissue located 2-5 cm from the tumor lesion. The immunohistochemical procedure was as follows.

Slide Preparation: Slides were soaked in concentrated sulfuric acid for 24 hours, rinsed thoroughly with tap water, dried at 60°C, and then coated with a 100 ml dilution of polylysine solution. They were left to dry naturally indoors.

Sectioning: Paraffin-embedded pathological slides were sectioned at 4 μm thickness and dried in a 60°C oven for 24 hours.

Dewaxing and Rehydration: Slides were dewaxed in xylene and rehydrated through graded alcohols (100%, 95%, 80%, 70%) for 5 minutes each, followed by rinsing in tap water for 3 minutes.

Antigen Retrieval: Using the EDTA method (pH 8.0), slides were placed in an autoclave on a metal rack, pressurized, and heated for 2 minutes, then cooled naturally in water.

Blocking: Slides were incubated in 3% H_2O_2 on a plastic rack at room temperature for 10 minutes to block endogenous peroxidase activity.

Primary Antibody Incubation: 70 μl of rabbit anti-human RSK4 antibody (EP1524Y, Abcam, Shanghai, China) diluted 1:100 was applied to each slide and incubated at 37°C for 1 hour or overnight at 4°C.

Secondary Antibody Incubation: 70 μl of ready-to-use fast immunohistochemical secondary antibody was applied and incubated at room temperature for 20 minutes.

Detection: Slides were developed with DAB solution and monitored under a microscope for color development. After achieving desired staining, slides were rinsed in running tap water for 5 minutes.

Counterstaining and Mounting: Slides were counterstained with hematoxylin for 2 minutes, differentiated with hydrochloric acid alcohol, rinsed, and then dehydrated through graded alcohols. Slides were cleared, mounted with neutral gum, and dried.

Judgment criteria

Cell staining was observed at 200X magnification. The proportion of positive cells was categorized into four grades: 0 (0-10%), 1 (10-25%), 2 (25-50%), and 3 (50-100%). The intensity of

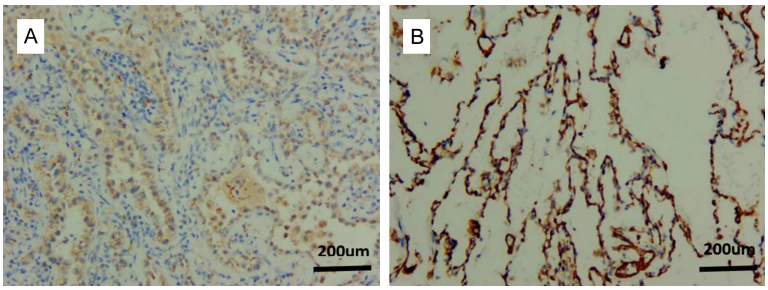

Figure 1. RSK4 protein expression in lung cancer (A) and adjacent tissues (B).

Table 1. Positive RSK4 protein expression in lung cancer tissues and adjacent cancer tissues

Organization	Positive	Proportion	Negative	Proportion
Carcinoma tissue (n = 100)	35	35.00	65	65.00
Adjacent tissue (n = 100)	69	69.00	31	31.00
X^2		11.115		
P		< 0.001		

staining was similarly graded: 0 for negative expression, 1 for light yellow cytosolic staining, 2 for brown cytosolic staining, and 3 for intense brown staining. The final score was calculated by multiplying the proportion and intensity grades. Interpretation of results was as follows: 0 indicated negative expression (-), 1-2 indicated weak positive expression (+), 3-4 indicated moderate positive expression (++), and scores above 4 indicated strong positive expression (+++). A score of 3 or higher was considered a positive result. Decisions were confirmed by consulting two experienced associate chief physicians in the pathology department [10].

Statistical analysis

Data were analyzed using SPSS 18.0 (SPSS Inc., Chicago IL USA). Measurement data, conforming to normal distribution, were expressed as mean ± standard deviation (x±sd) and analyzed using the t-test. Categorical data were presented as [n (%)] and analyzed using the χ^2 test or Fisher's exact test. Differences in RSK4 protein expression between lung cancer and adjacent tissues were assessed using paired chi-square test (McNemar's test). The association of RSK4 protein expression with risk factors and prognosis was examined using Spearman's correlation. Multivariate Cox regression was utilized to identify risk factors for positive RSK4 protein expression. A *p*-value < 0.05 was considered statistically significant.

Results

Positive RSK4 protein expression in lung cancer tissues and adjacent cancer tissues

The typical immunization results of positive RSK 4 protein in lung cancer tissues and adjacent cancer tissues are shown in **Figure 1**. The positive expression rate of RSK4 protein in lung cancer tissues was 35.00%, significantly lower than the 69.00% in adjacent tissues, indicating a significant difference (P < 0.05), as shown in **Table 1**.

Differences in RSK4 protein expression across clinical features

Analysis of RSK4 protein expression across different clinicopathological features showed significant differences primarily in TNM stage, tumor differentiation, and lymph node metastasis. Lower expression rates were noted in patients with advanced TNM stage, poor differentiation, and lymph node metastasis compared to patients at stage I+, with moderate to high differentiation and no lymph node metastasis (all P < 0.05). No significant differences in RSK4 protein expression were observed in patients with other clinical characteristics (all P > 0.05), as detailed in **Table 2**.

Multivariate Cox regression analysis of factors affecting RSK4 protein expression

The scale of the multivariate analysis is presented in **Table 3**, with positive RSK4 protein occurrence as the dependent variable. Independent variables included TNM staging, differentiation, and lymph node metastasis. The selection of the model was based on actual clinical scenarios. Results from the Cox regression analysis indicated that advanced TNM stage, poor differentiation, and the presence of lymph node metastases significantly impacted RSK4 protein expression (all P < 0.05). These findings are detailed in **Table 4** and **Figure 2**.

Prognosis of patients with positive and negative RSK4 protein expression

Follow-up records were analyzed to compare the prognostic outcomes between patients

Table 2. Differences in RSK4 protein expression across clinical features

Metric	Example number	Positive test (n = 35)	Negative (n = 65)	X^2	*P*
Age				0.733	0.392
≥ 60	40	16	24		
< 60	60	19	41		
Sex				0.029	0.866
Male	56	20	36		
Female	44	15	29		
Smoke				0.544	0.461
Yes	55	21	34		
No	45	14	31		
The maximum diameter of the tumor				0.233	0.630
≥ 3 cm	51	19	32		
< 3 cm	49	16	33		
TNM by stages				5.202	0.023
I+II designated time	56	25	31		
III designated time	44	10	34		
Differentiation situation				6.564	0.010
High differentiation	57	26	31		
Poorly differentiation	43	9	34		
Lymphatic metastasis				4.574	0.032
Yes	57	25	33		
No	43	10	32		

Table 3. Value scale for multivariate analysis

Factor	Assignment
Dependent Variable	Y
RSK4 protein expression	Positive = 0; negative = 1
Independent variable	X
TNM by stages	I+II designated time = 0; III designated time = 1
Degree of differentiation	Medium to high differentiation = 0; low score cost = 1
Lymphatic metastasis	No = 0; Yes = 1

with positive and negative RSK4 protein expression. The survival rate for patients with positive expression was 74.29% (26 out of 35), which was higher than the 53.85% (35 out of 65) observed in patients with negative expression, as illustrated in **Figure 3**.

Discussion

RSK is a 90 kD intracellular serine/threonine protein kinase, known as p90RSK or RSK, which phosphorylates the 40S ribosomal subunit S6 to enhance the translation of specific mRNAs. This kinase activity is further regulated through serine/threonine phosphorylation within its own molecule [11-13]. RSK proteins share a basic structure consisting of two functional kinase domains: the N-terminal kinase domain and the C-terminal kinase domain, connected by a junction region. Variations in the terminal and junction regions may determine the distinct functions of each subtype. RSK4, an X-linked gene, plays a significant role in cancer, as X-linked oncogenes and tumor suppressor genes are regulated at the gene level, and their overexpression might correlate with X chromosome copy number alterations [14]. Furthermore, RSK4 is highly expressed in tissues such as the brain, heart, kidney, and skeletal muscle, but is notably absent in normal lung tissue, as documented in reference [15].

Table 4. Multivariate Cox regression analysis of factors affecting RSK4 protein expression

Metric	Results of the multivariate analysis				
	β	*Wald/X²*	*OR*	95% CI	*P value*
TNM by stages	0.300	40.215	0.551	0.351-0.913	0.005
Differentiation	0.232	7.841	0.661	0.387-0.846	0.002
Lymphatic metastasis	0.524	11.136	0.532	0.216-0.795	0.001

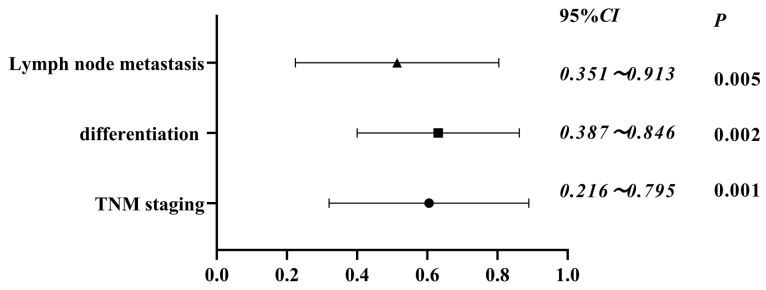

Figure 2. Multivariate Cox regression analysis of factors affecting RSK4 protein expression.

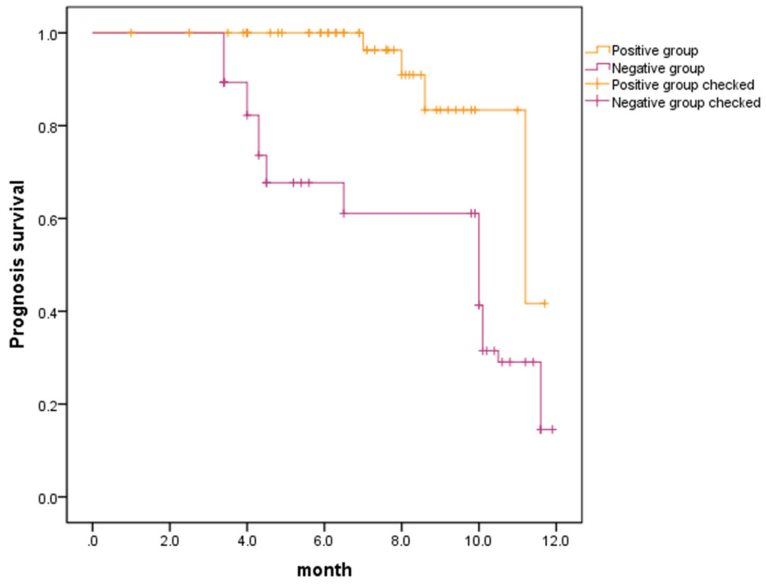

Figure 3. Prognostic status of patients with both positive and negative RSK4 protein.

In this study, immunohistochemical staining was utilized to evaluate RSK4 protein expression in NSCLC and adjacent tissues. The positive expression rate of RSK4 in NSCLC tissues was 35.00%, significantly lower than the 69.00% observed in adjacent tissues, suggesting a potential tumor suppressor role for RSK4 in NSCLC. Previous research has shown that methylation of the RSK4 promoter in NSCLC and breast cancer may silence RSK4 expression, potentially contributing to the loss of its tumor-suppressive effects [16].

Further analysis of RSK4 protein expression in relation to clinical data revealed significantly lower positive expression rates in cases with advanced TNM stage, poor differentiation, and lymph node metastasis compared to those at stage I+, with moderate to high differentiation and no lymph node metastasis. Cox regression model results confirmed that these factors significantly affect RSK4 protein expression, indicating RSK4's involvement in the progression and metastasis of NSCLC. This involvement may be mediated by RSK4's ability to activate tight junction proteins and inhibit chemokine receptor expression, thus reducing cell metastasis and chemotaxis [17]. Additionally, RSK4 might influence the p53 signaling pathway by upregulating p21 expression, promoting tumor cell aging and apoptosis. Notably, RSK4 expression has also been found to decrease significantly in colon adenoma tissues prior to colon carcinogenesis, suggesting early alterations or loss of RSK4 during tumorigenesis [18].

The expression of RSK4 protein is reduced in NSCLC tissues, correlating with the malignant proliferation of tumor cells. This study confirms that a higher RSK4 expression is significantly associated with better prognosis, with survival rates of 74.29% (26 out of 35) in the positive expression group versus 53.85% (35 out of 65) in the negative group. Spearman correlation analysis revealed that positive RSK4 protein expression is significantly negatively correlated with TNM

stage and lymph node metastasis, yet shows a significant positive correlation with patient prognosis. These findings suggest that RSK4 protein expression levels are indicative of a benign prognosis in lung cancer patients.

Additionally, RSK4 protein expression in NSCLC tissues has been linked to epithelial-to-mesenchymal transition and the likelihood of malignant tissue transformations. This protein also influences cell motility, leading to variations in cell morphology that could affect tumor staging. Moreover, RSK4 may impair lymphangiogenic factors, thereby participating in NSCLC progression [19]. RSK4 is also thought to enhance tumor cell activity and actin fiber dynamics in NSCLC, facilitating morphological changes that allow tumor cells to evade immune surveillance and promote local proliferation and distant metastasis, consequently reducing postoperative survival times [20].

There are still some limitations in this study. Being retrospective, this study may contain biases due to possible errors in patient data collection, which could affect the results. Future studies should consider expanding the sample size and adopting a prospective approach to further explore the expression of RSK4 protein in NSCLC tissues, adjacent non-tumor tissues, and its correlation with clinical and pathological features.

The positive expression of RSK4 in NSCLC tissues and its significant correlation with cancer stage, differentiation, and lymph node metastasis suggest its role as a tumor suppressor gene, closely associated with patient prognosis.

Disclosure of conflict of interest

None.

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