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Original contribution

Overexpression of RCN1 correlates with poor prognosis and progression in non–small cell lung cancer☆,☆☆

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Summary We investigated the expression of reticulocalbin-1 (RCN1) and its prognostic significance in non–small cell lung cancer (NSCLC). RCN1 expression was evaluated by immunohistochemical analysis with tissue microarrays in NSCLC tissues and matched adjacent noncancerous tissues. Furthermore, quantitative polymerase chain reaction and Western blot were also used to examine the expression of RCN1. Moreover, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, clone formation, and transwell assays were used to measure cell proliferation, migration, and invasion. Lastly, we used the Kaplan-Meier method and log-rank test to compare overall survival rates between the RCN1–high expression group and the RCN1–low expression group. In this study, immunohistochemistry by tissue microarray at RCN1 expression was significantly up-regulated in NSCLC tissues compared with adjacent noncancerous tissues. We further confirmed the up-regulation of RCN1 by quantitative polymerase chain reaction and Western blot assay. RCN1 expression level was closely related to lymph node metastasis ($P < .001$) and TNM stage ($P = .012$). Kaplan-Meier analysis showed that high RCN1 expression was remarkably associated with poor prognosis with NSCLC patients. A suppression of cell proliferation, migration, and invasion was obtained in A549 cells treated with RCN1 small interfering RNA. Our data indicate that RCN1 expression may have an vital role at promoting the occurrence of NSCLC, and it may be a vital molecular marker in the diagnosis and prognosis of NSCLC patients.

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1. Introduction

Lung cancer is the main reason of cancer-related deaths worldwide [1]. Accordingly, non–small cell lung cancer (NSCLC) accounts for nearly 80% of lung cancers. Although the incidence rate of NSCLC is decreasing with improvement of early diagnosis and treatment modalities, its 5-year survival rate remains very low [2]. Hence, it is urgently essential to identify the underlying molecular mechanism and search novel well-characterized biomarkers to improve clinical outcome of patients with NSCLC [3].

Reticulocalbin-1 (RCN1) is a Ca^{2+}-binding protein discovered 2 decades ago and found to be stored in endoplasmic reticulum [4]. It is speculated that the function of RCN1 has played a role in Ca^{2+}-dependent cell adhesion [5], because dysregulation of RCN1 protein has been reported in multifarious diseases, including cancer, cardiovascular, and neuromuscular diseases [6-8]. An increasing number of reports indicate that up-regulation of RCN1 was found in multiple tumorous types, such as breast cancer [7], colorectal cancer [5], liver cancer [9], and kidney cancer [10]. These findings indicate that overexpression of RCN1 in cancers could contribute to its role as an oncogene in tumorigenesis and tumor progression.

Here, we reported that the expression of RCN1 was up-regulated and dramatically correlated with aggressive clinicopathological characteristics in NSCLC. Moreover, we investigated the effects of RCN1 expression on cell proliferation, migration, and invasion in NSCLC cell lines. Together, these results identify RCN1 as an independent prognostic factor in NSCLC, identifying it as a potential therapeutic target in NSCLC.

2. Materials and methods

2.1. Patients and tissue specimens

We collected formalin-fixed, paraffin-embedded NSCLC samples and matched adjacent noncancerous tissues from 158 patients who underwent surgery at the Affiliated Hospital of Nantong University from 2010 to 2015. None of the patients received prior chemotherapy or radiotherapy. Clinicopathological data, including patient sex, age, smoking status, tumor size, histologic type, tumor status, lymph node metastasis, distant metastasis, and TNM stage, were obtained. Survival time was defined as the time from operation to the time of the last follow-up day or patient mortality. Informed consent has been obtained from all patients, and the study protocol was approved by the Research Ethics Committee of Affiliated Hospital of Nantong University.

2.2. Immunohistochemistry

We used tissue microarray (TMA) system in the Department of Pathology, Affiliated Hospital of Nantong University. Core tissue biopsies (2 mm in diameter) were taken from individual paraffin-embedded sections and settled in recipient paraffin blocks. TMA specimens were cut into 4-μm sections and mounted on superfrost-charged glass microscope slides. We used TMA analysis as a quality control for hematoxylin and eosin staining. Then tissue sections were deparaffinized and rehydrated in graded ethanol. Antigen retrieval was carried out by boiling sections in ethylenediaminetetraacetic acid buffer at pH 6.0 for 3 minutes. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 30 minutes. TMA slides were stained using rabbit monoclonal anti-RCN1 antibody (ab184441; Abcam, Cambridge, UK) at 4°C overnight and then incubated with a horseradish peroxidase–conjugated antirabbit secondary antibody (Sigma-Aldrich, St Louis, MO) at 37°C for 30 minutes. Slides were then processed using horseradish peroxidase and 3,3′-diaminobenzidine chromogen solution and counterstained with hematoxylin.

Immunohistochemistry score (IHS) was determined by evaluating staining intensity and density. Briefly, RCN1 expression levels were scored semiquantitatively according to the percent of positively stained cells combined with the staining intensity. The immumohistochemical staining results were evaluated by 3 observers independently, and similar results came out. The grade was according to the number of stained cells and the staining intensity of the individual cells. The staining intensity was scored as follows: 0 (negative), 1 (weakly positive), 2 (moderately positive), and 3 (strongly positive). The percentage of RCN1-positive cells was also scored as follows: 1 was given for 0% to 10%, 2 for 11% to 50%, 3 for 51% to 80%, and 4 for 81% to 100%. Multiplying every observer's score, we calculated the total score, which theoretically ranged from 0 to 12. A total score of 0 was considered negative (−); 1 to 3, as (+); 4 to 8, as (++); and 9 to 12, as (+++). Samples with IHS ≤ 3 were considered to show low RCN1 expression, whereas those with IHS ≥ 4 were considered to exhibit high RCN1 expression.

2.3. Cell lines and cell culture

Human NSCLC cell lines A549, H460, H1229, and H358 and human bronchus epithelial cell line Beas-2b were all purchased from the American Type Culture Collection (Rockville, MD) and grown in Dulbecco modified Eagle medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 IU/mL penicillin-streptomycin mixture (Sigma-Aldrich). Cells were cultured at 37°C in a 5% CO_2 atmosphere in a humidified incubator.

2.4. Quantitative real-time polymerase chain reaction

Total RNA was extracted from tumor tissue samples using TRIzol (Gibco, Waltham, MA). Two micrograms of total RNA was subjected to DNaseI digestion (Fermentas, Hanover, MD) at 37°C for 30 minutes and then to heat inactivation of DNaseI at 65°C for 10 minutes. Messenger RNA (mRNA)

level was tested using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). β-Actin was used as an internal control. The primers are as follows: *RCN1* gene 5′-CAACCAGAGCTTCCAGTACGA-3′ (sense) and 5′-TTTCTGCACCCGTTTGATCCA-3′ (antisense). All reactions were performed on a LightCycler480 (Roche Diagnostics, Mannheim, Germany) using the following cycling parameters: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and

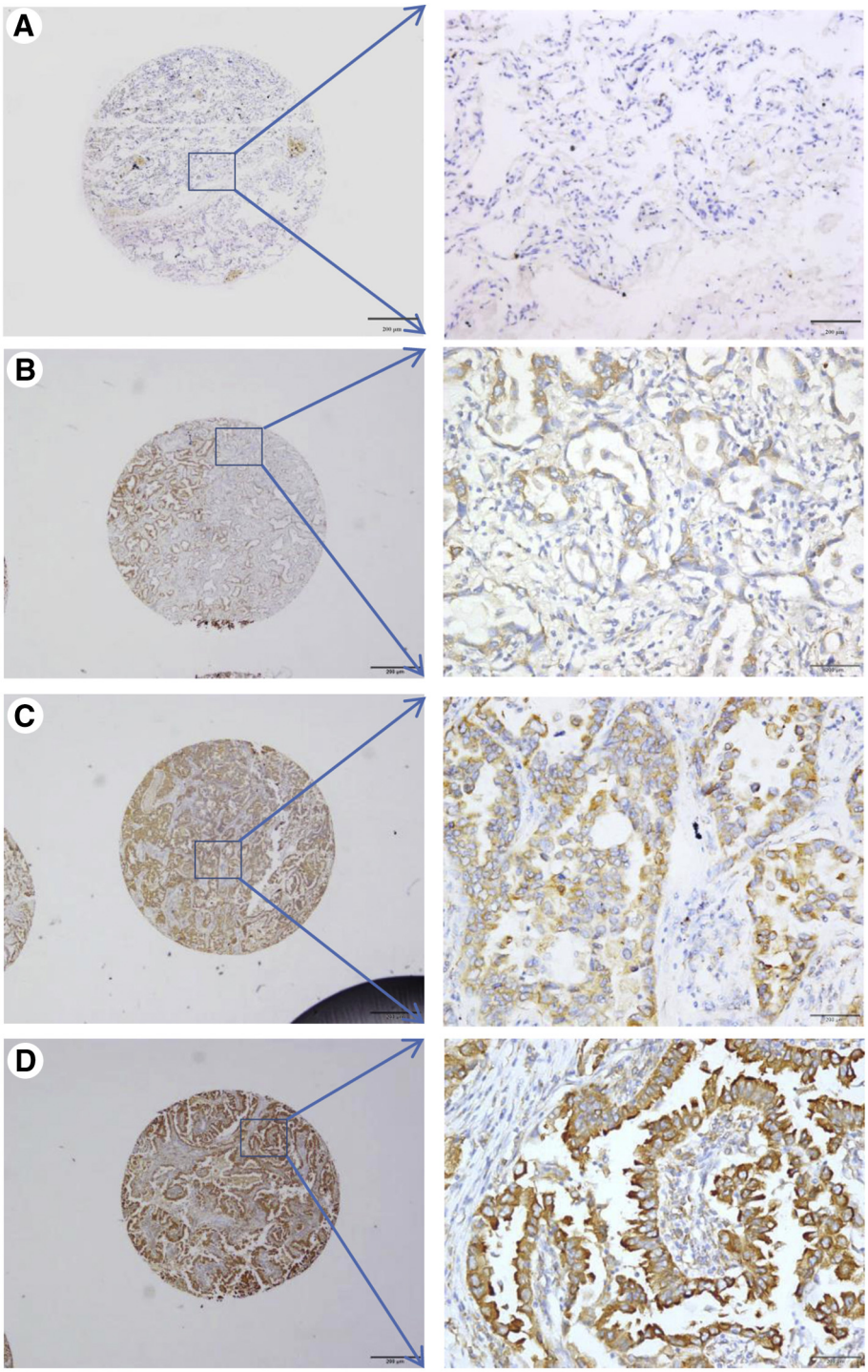

Fig. 1 Expression of RCN1. Representative photographs of RCN1 expression in NSCLC and adjacent noncancerous tissue. A, Negative staining in adjacent noncancerous tissues, scored as RCN1−. B, Weak staining in well-differentiated NSCLC tissues, scored as RCN1+. C, Moderate staining in moderately differentiated NSCLC tissues, scored as RCN1++. D, Strong staining in poorly differentiated NSCLC tissues, scored as RCN1+++.

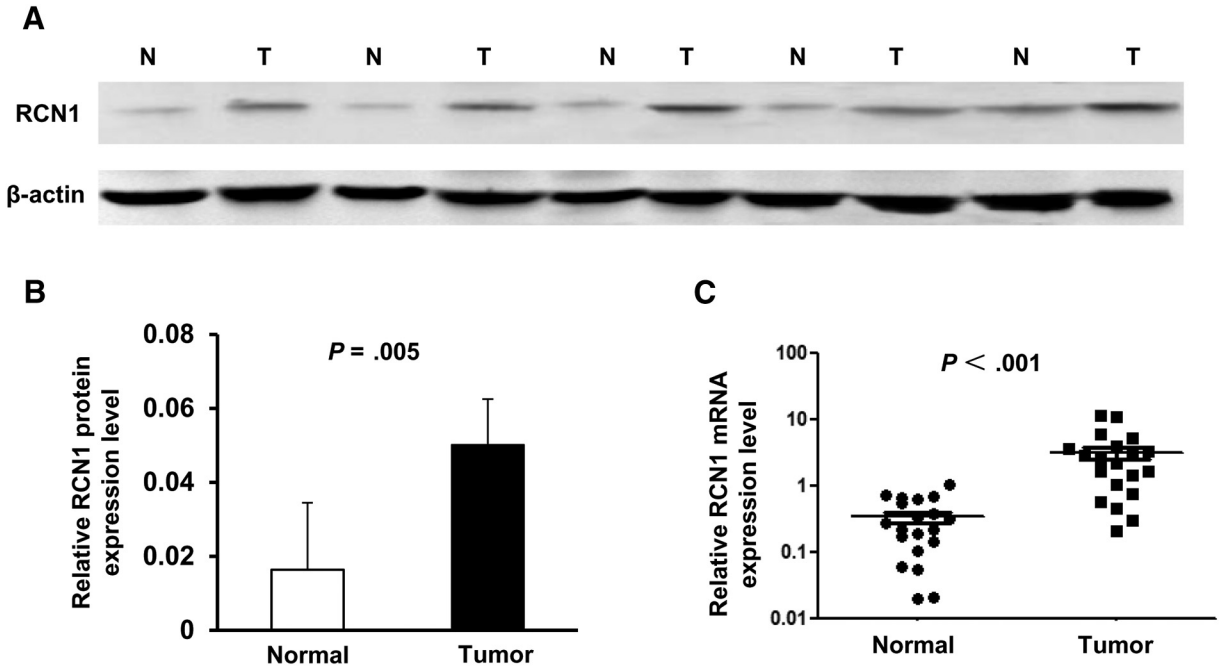

Fig. 2 Relative expression of RCN1 in NSCLC. A, Western blot analysis of 5 representative paired tissue samples of NSCLC (T) and their matched adjacent noncancerous tissues (N). B, Relative average expression levels of RCN1 protein were remarkably up-regulated in 20 (83.3%) of 24 NSCLC tissues compared with the matched adjacent nontumorous tissues ($P = .005$). C, qPCR was performed to show mRNA expression levels of RCN1 in NSCLC compared with cancer-adjacent tissues. Relative expression levels of RCN1 mRNA in NSCLC were significantly higher than those in matched nontumorous tissues ($P < .001$).

Table 1 Clinicopathological correlation of RCN1 expression in NSCLC patients

Clinical parameters	Total (n = 158)	RCN1 expression		*P*
		Low level (n = 66)	High level (n = 92)	
Sex				.249
Male	97	44	53	
Female	61	22	39	
Age (y)				.980
<60	86	36	50	
≥60	72	30	42	
Tumor diameter (cm)				.218
<3	39	13	26	
≥3	119	53	66	
Smoking status				.056
Smokers	84	41	43	
Nonsmokers	74	25	49	
Histologic type				.325
Adenocarcinoma	91	35	56	
Squamous cell carcinoma	67	31	36	
Lymph node metastasis				<.001 *
Positive	73	18	55	
Negative	85	48	37	
TNM stage				.012 *
I/II	107	52	55	
III/IV	51	14	37	
Distal metastasis				.801
Absent	150	63	87	
Present	8	3	5	

* $P < .05$ was considered statistically significant.

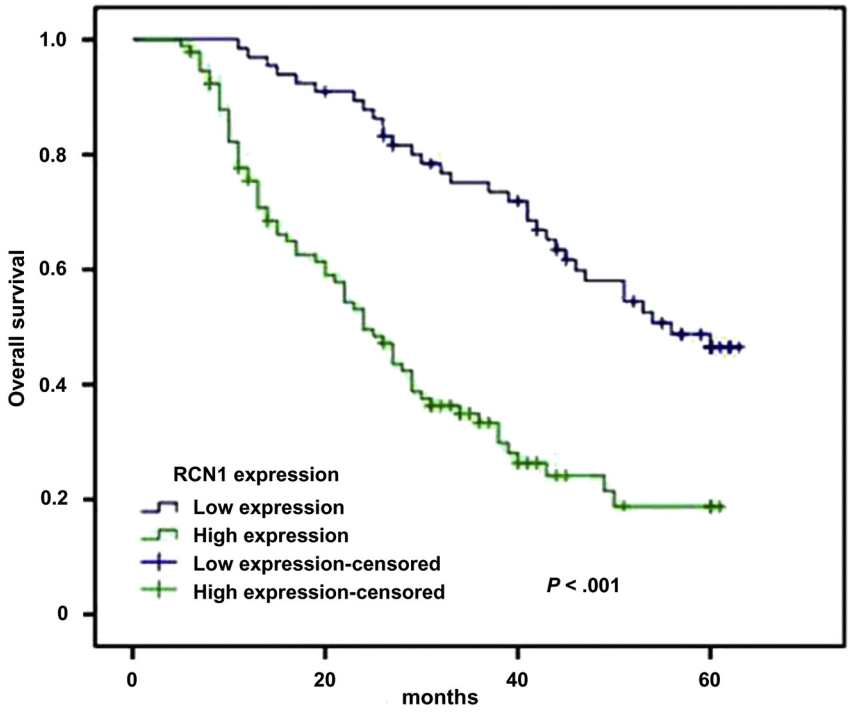

Fig. 3 Kaplan-Meier method was used to evaluate the survival analysis of NSCLC patients. Patients with high RCN1 expression had significantly worse overall survival rate than did patients with low RCN1 expression ($P < .001$).

60°C for 45 seconds. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

2.5. Western blot

Fresh tissues and cell extracts were prepared with a cell lysis reagent (Sigma-Aldrich) according to the manufacturer's instructions, and the protein was quantified by a BCA assay (Pierce, Rockford, IL). Moreover, the protein samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10%) and detected by using rabbit monoclonal anti-RCN1 antibody (ab184441; Abcam). Goat antirabbit IgG (Pierce) secondary antibody conjugated to horseradish peroxidase and ECL detection systems (SuperSignal West Femto; Pierce) were used for detection.

2.6. Cell proliferation assay

Cells were seeded in 96-well plates at 5×10^3 per well at the indicated times after transfection and were assayed using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) kit (Promega, Madison, WI). Then 20 μL of MTT assay solution was added into 100 μL of medium containing cells and incubated for 2 h. After supernatants were removed, the formazan crystals were dissolved in 100 μL/well dimethylsulfoxide. The absorbance of each well was detected using a

Table 2 Cox proportional hazards model analysis of prognostic factors

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*	HR	95% CI	*P*
RCN1 expression (low *vs* high)	2.115	1.023-4.232	.023 *			<.001 *
Sex (male *vs* female)	1.425	0.740-2.686	.385	–	–	–
Age (<60 y *vs* ≥60 y)	1.925	0.882-3.562	.332	–	–	–
Tumor diameter (<3 cm *vs* ≥3 cm)	1.456	0.564-2.954	.440	–	–	–
Smoking status (smokers *vs* non-mokers)	1.145	0.365-3.224	.823	–	–	–
Histologic type (adenocarcinoma *vs* squamous cell carcinoma)	2.124	0.685-3.947	.058	–	–	–
TNM stage (III + IV *vs* I + II)	1.345	0.694-2.498	.047 *	1.210	0.587-2.449	.038 *
Lymph node metastasis (positive *vs* negative)	1.183	0.618-2.380	.004 *	1.382	0.596-2.732	.014 *
Distal metastasis (absent *vs* present)	1.392	0.545-3.212	.741	–	–	–

Abbreviations: CI, confidence interval; HR, hazard ratio.
* $P < .05$ was considered statistically significant.

microplate reader (Molecular devices, Sunnyvale, CA) at 490 nm. All assays were performed and repeated 3 times.

2.7. Transfection of small interfering RNA

For small interfering RNA (siRNA) silencing of RCN1, RNA interference assay was performed by using synthetic siRNA duplexes. According to sequences of human RCN1 gene sequence (NM_002901), 3 specific siRNAs targeting RCN1 were designed and synthesized from GenePharma (Shanghai, China). The targeting sequences were as follows: siRNA1, 5′-GGAUGAGAAGCUAACUAAAGA-3′; siRNA2, 5′-GGACGGGAAGUUAGACAAAGA-3′; and siRNA3, 5′-GGAAGUUAGACAAAGAUGAGA-3′. A nontargeting scramble sequence was transfected as a negative control. siRNAs or scramble was transfected into cells with 400 pmol, respectively, using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. After 24 hours of transfection, cells were harvested for cell proliferation, migration, invasion, and colony formation assays. Both siRNA2 and siRNA3 could effectively reduce endogenous RCN1 expression by Western blotting assay, and the siRNA3 has the best inhibition effect. Therefore, the siRNA3 was chosen for downstream experiment.

2.8. Colony formation assay

Cells (5×10^4/well) were seeded in a 24-well plate after transfection. After 24 hours, the cells were collected and seeded (1000/well) in a 6-well plate for 12 days. Surviving colonies (>50 cells per colony) were counted after fixing with methanol/acetone (1:1) and staining with 5% Gentian Violet (ICM Pharma, Singapore, Singapore). After washing 3 times with phosphate-buffered saline to cut away extra dyes, the colonies were photographed and counted. The assay was executed in triplicate wells.

2.9. Transwell chamber migration and invasion assay

The upper well of the transwell (Corning Inc, Corning, NY) was coated with Matrigel (BD Biosciences, San Jose, CA) at 37°C in a 5% CO_2 incubator for 1 hour. These cells were serum starved for 24 hours, and then 500 μL of cell suspension containing 10^5 cells/mL was placed in the upper compartment of the chamber. Culture medium supplemented with 10% fetal bovine serum was added into the lower well of the chamber. The plates were incubated for 48 hours. At the end of the incubation, the cells on the upper surface of the filter were completely removed by wiping with a cotton swab. Cells that migrated and invaded into the lower well were washed with phosphate-buffered saline, fixed in 4% paraformaldehyde, and stained by 0.2% crystal violet. The numbers of cells were photographed and counted under microscopy. Each assay was carried out in triplicate.

2.10. Statistical analysis

All statistical analyses were performed using SPSS17.0 statistical software (SPSS Inc, Chicago, IL). A χ^2 test was conducted to test the correlation of expression of RCN1 with clinicopathological variables. The survival curves were

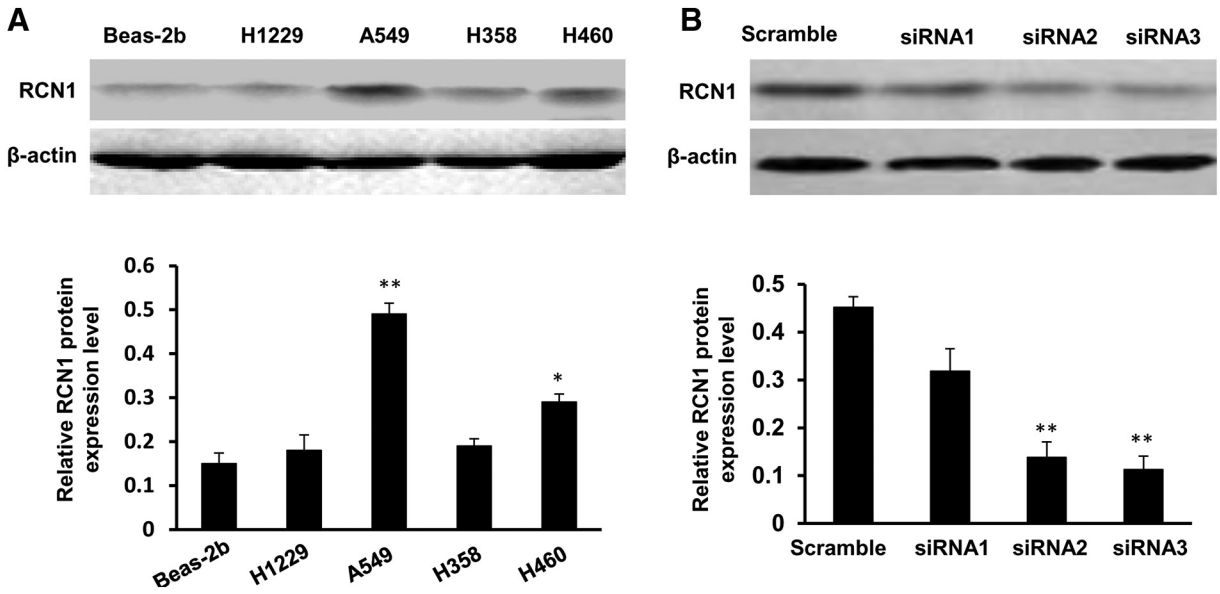

Fig. 4 The expression of RCN1 in NSCLC cell lines. A, Western blot analysis showed the RCN1 protein expression levels in 4 NSCLC cell lines and human bronchial epithelial cell line BEAS2B. *$P < .05$ versus Beas-2b, **$P < .01$ versus Beas-2b. B, Western blot analysis exhibited RCN1 protein expression levels after transfection of the 3 different RCN1 siRNA transfectants (siRNA1, siRNA2, and siRNA3) and the Scramble. **$P < .01$ versus Scramble.

calculated using the Kaplan-Meier method, and the log-rank test was used for survival analysis. Cox proportional hazards model was used for multivariate analyses of the prognostic values. $P < .05$ is considered statistically significant.

3. Results

3.1. Evaluation of RCN1 expression in NSCLC

Using TMA-based immunohistochemistry studies, we examined the expression of RCN1 protein in collected paraffin-embedded tissue samples from 158 NSCLC patients. The results showed that RCN1 was expressed at various levels in NSCLC tissues and the matched noncancerous tissues (Fig. 1). Positive staining was predominantly localized in the cytoplasm of NSCLC cells. Among the 158 samples, high RCN1 expression was found in 58.2% (92/158) of NSCLC tissues. Relative expression of RCN1 mRNA in NSCLC (n = 20) and adjacent nontumorous tissues (n = 20) were quantified by quantitative polymerase chain reaction (qPCR). The results showed that the average RCN1 mRNA expression level was significantly higher in NSCLC tissues compared with nontumorous tissues ($P < .001$; Fig. 2C). To further confirm the results of qPCR, we measured RCN1 expression in 20 NSCLC tissues and matched adjacent noncancerous tissues by Western blot. The representative Western blot results in 6 cases are shown in Fig. 2A. The results show that RCN1 protein level was up-regulated in the NSCLC tissues (80%; 16/20) compared with matched adjacent normal lung tissues. The average RCN1 protein expression level in 20 NSCLC tissues was significantly higher than that in corresponding adjacent noncancerous tissues ($P = .005$; Fig. 2B).

3.2. The correlations between the expression of RCN1 and various clinicopathological characteristics

Moreover, we analyzed the relationship between the degree of RCN1 immunohistochemical staining and various clinicopathological characteristics by using the χ^2 analysis. We showed that the RCN1 expression level in cancer tissues was significantly associated with clinicopathological features,

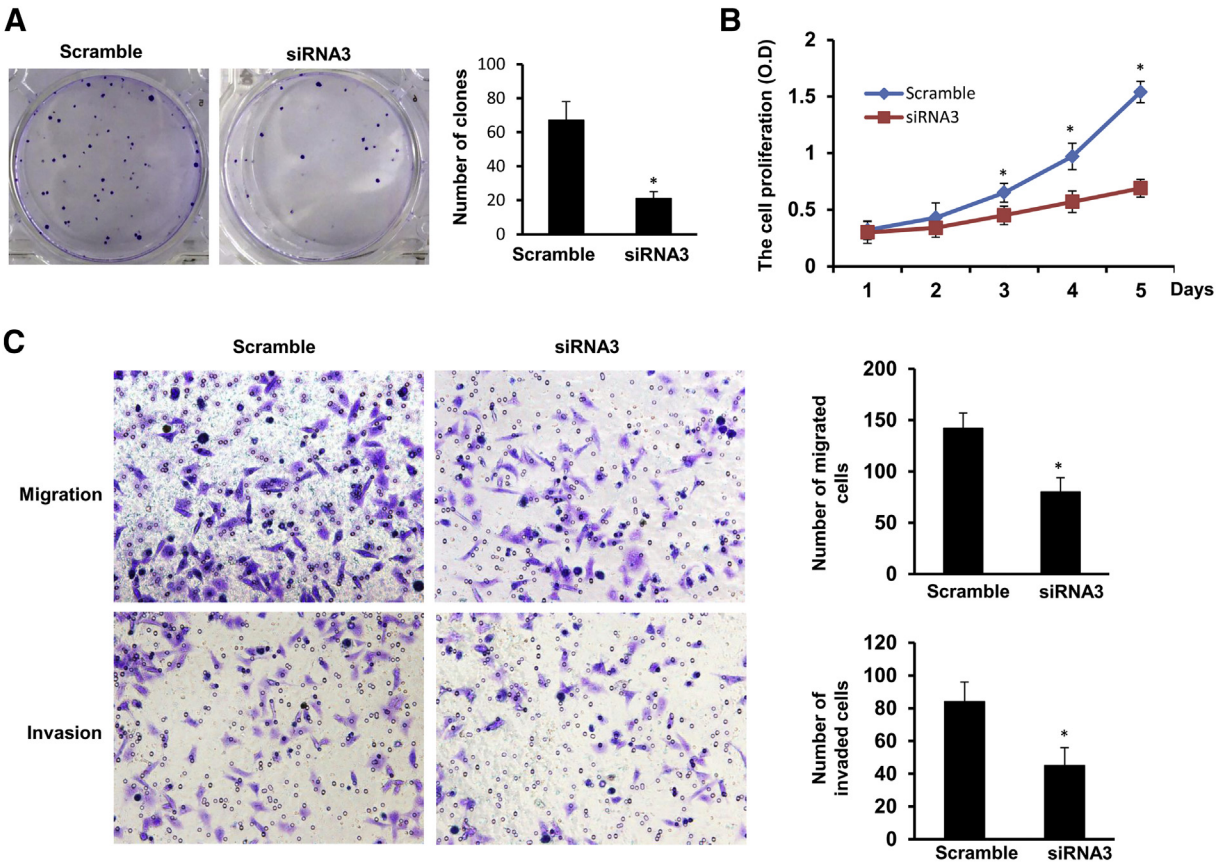

Fig. 5 Knockdown of RCN1 expression inhibited NSCLC cell proliferation, viability, migration, and invasion by using siRNA. A, Colony numbers of the A549/siRNA and control cells were shown in plate colony formation assays. B, Cell proliferation (optical density) was performed with MTT assay using absorbance readings at 490 nm. A549 cells were transfected with RCN1 siRNA or Scramble for 1, 2, 3, 4, and 5 days, respectively. The values are evaluated by the mean of 3 determinations. C, Transwell assays showed that knockdown of RCN1 expression by siRNA inhibited cell migration and invasion in A549 cells. Original magnification ×100. *$P < .05$ versus Scramble.

including TNM stage and lymph node metastasis, but not with sex, age, tumor size, smoking status, histologic type, and distal metastasis (Table 1).

3.3. Prognostic value of RCN1 expression

The prognostic significance of RCN1 in NSCLC patients was evaluated by survival analysis of the high- and low-level expression groups of RCN1. The patients with low RCN1 expression group had a significantly longer overall survival than did those with high expression ($\chi^2 = 26.752$; $P < .001$, log-rank test; Fig. 3). Univariate Cox regression analysis showed that RCN1 expression, TNM stage, and lymph node metastasis were markedly correlated with the overall survival of NSCLC patients. Meanwhile, multivariate Cox regression analyses indicated that RCN1 expression, TNM stage, and lymph node metastasis can be used as independent predictors for overall survival of RCN1 patients (Table 2).

3.4. Down-regulation of RCN1 expression inhibited cell proliferation, migration, and invasion abilities

For research on the effects of RCN1 on the viability, proliferation, migration, and invasion of NSCLC cells, we hypothesized that RCN1 played a role as a cancer-promoting gene by accomplishing the above-mentioned study. We used the MTT and colony formation assays to identify the effect of RCN1 on cell viability and proliferation. A549 cells have the highest expression of RCN1 in NSCLC cells (Fig. 4A). After transfection with RCN1 siRNA1, RCN1 siRNA2, and RCN1 siNRA3, we found that siRNA1 reduced the level of endogenous RCN1 expression more dramatically than did siRNA2 and siRNA3 by Western blot (Fig. 4B). The results demonstrated that the viability of A549 cells was lower and the number of colonies of A549 cells in the siRNA-treated group was less than that in the control group (Fig. 5A; $P < .05$). Also, MTT assay showed that knockdown of RCN1 inhibited the proliferation of A549 cells (Fig. 5B; $P < .05$). Besides, transwell assay shows that knockdown of RCN1 expression significantly decreased the migrated and invaded cell number of A549 cell lines (Fig. 5C; $P < .05$).

4. Discussion

Lung cancer mortality has been gradually increasing over the past decades and is the second leading cause of death in China [11]. Thus, most efforts have been made to find sensitive and specific molecular markers that predict prognosis and act as therapeutic targets [12]. As far as we know, this study provides the first report of alteration of RCN1 expression and its prognostic impact in patients with NSCLC. RCN1, an endoplasmic reticulum–located calcium-binding protein, contains 6 conserved domains with similarity to a high-affinity calcium-binding motif [4]. Up-regulation of RCN1 has also been reported in several tumor cells [13]. Recent studies identified that RCN1 is also localized at the cell surface of prostate cancer cells [14]. Previous studies revealed that RCN1 is highly expressed in an invasive breast cancer cell line and an invasive colorectal cancer cell line, MDA-MB-435 and SW480, respectively [5,7], strongly suggesting that RCN1 is implicated in tumor cell invasiveness. Moreover, RCN1 was overexpressed in lung cancer tissues compared with normal lung tissues, as well as being highly expressed in lymphatic endothelial cells in cancers compared with noncancer tissues. Thus, RCN1 has been evidenced as a significant lymphatic endothelial cancer marker that is capable of controlling lymphatic metastasis [13]. Furthermore, RCN1 has also been confirmed as a potential renal cell carcinoma cancer marker in clinical proteomic analysis [10]. Strong RCN1 expression associates with a low expression of Ca^{2+}-dependent cadherin in colorectal cancer cells [5]. Especially, RCN1 was regarded as a prognostic factor to measure postoperative adjuvant chemotherapy responders in NSCLC [15]. These above observations imply that RCN1 expression involved in cancer development and progression. Hence, we speculate that strong expression of RCN1 correlates with decreased expression of E-cadherin, leading to increased invasiveness of cancer cells [7].

In our previous study, we found that expression of RCN1 was up-regulated at the mRNA and protein levels, respectively, in most NSCLC tissues compared with matched adjacent noncancerous tissues using qPCR and Western blot analysis. Moreover, TMAs with NSCLC specimens and immunohistochemistry analysis indicated higher RCN1 protein expression in NSCLC tissues than in matched adjacent noncancerous tissues, which is consistent with previous findings concerning high RCN1 expression in various malignancies. However, our study shows that RCN1 is also located in the cytoplasm. Furthermore, high RCN1 expression in NSCLC correlated with some clinicopathological parameters, such as lymph node metastasis and TNM stage. In addition, Kaplan-Meier analysis demonstrated that high RCN1 expression was dramatically associated with shorter survival time of NSCLC patients. Univariate analysis revealed that RCN1 expression, TNM stage, and lymph node metastasis were correlated with overall survival of NSCLC patients. Also, multivariate analysis further showed that RCN1 expression, TNM stage, and lymph node metastasis were independent prognostic factors. Moreover, knockdown of RCN1 expression inhibited the viability, proliferation, and migration and invasion of A549 cells. Taken together, these above results indicated that RCN1 plays a crucial role in the growth and metastasis of NSCLC.

5. Conclusions

We have shown that RCN1 was up-regulated in NSCLC tissues and significantly associated with lymph node metastasis and TNM staging. Moreover, high RCN1 expression correlated with poorer overall survival, indicating a predictive value

in NSCLC. Taken together, these results comprehensively demonstrated the biological effects of RCN1 in NSCLC carcinogenesis, as well as the acceleration of cell proliferation, migration, and invasion, which might serve as potential biomarkers or therapeutic targets for NSCLC treatment.

Authors' contributions

X. C. and W. S. performed the molecular biological assay. X. C. provided professional writing, and H. H. and X. F. contributed to data analysis. S. Y. and H. K. contributed to the design of the study and critically reviewed the manuscript. All authors read and approved the final manuscript.

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