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Original Article

PRR14 is an independent predictor of poor prognosis in resected non-small cell lung cancer patients

He Yu*, Ping Zhou*, Dan Li, Dan Liu, Weimin Li

*Department of Respiratory Medicine, West China Hospital, Sichuan University, Chengdu, China. *Equal contributors.*

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Abstract: Recently PRR14 has been found to be involved in lung carcinogenesis, and the high-level mRNA expression of PRR14 is associated with worse survival overall in lung cancers. However, there are few studies focused on the problem. Therefore, we evaluated the expression of PRR14 by immmunohistochemistry and the associations with prognosis in resected non-small cell lung cancer (NSCLC) patients. Totally, a number of 199 patients were enrolled in our study. We found that PRR14 was nuclear staining in lung tumor samples and positive expression in 95 out of 199 NSCLC patients (47.7%). PRR14 expression was significantly associated with gender, smoking history, histological type, lymph node infiltration in resected NSCLC patients (all $P<0.05$). Patients with PRR14-positive expression had worse 5-year survival ($P=0.002$) and shorter progression-free survival (PFS, $P=0.006$) than patients with PRR14-negative expression by univariate analysis. More interestingly, the multivariate analysis also suggested that PRR14 positive expression was significantly related to poorer OS and PFS (all $P<0.05$), independent of the clinicopathological features of NSCLC patients. Thus, our study indicated that PRR14 was an independent predictor of unfavorable prognosis in resected NSCLC patients and may serve as a potential target.

Keywords: PRR14, non-small cell lung cancer, prognosis

Introduction

Lung cancer is one of the most frequently diagnosed carcinomas and the leading cause of cancer-related deaths worldwide [1]. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancer cases in the United States and about 18.7% of NSCLC patients can achieve 5-year survival [2]. Therefore, it is important to find some biomarkers to evaluate and improve prognosis of NSCLC patients.

The Proline-Rich Protein 14 (PRR14) is encoded by the proline rich 14 gene, which is located in the 16p11.2 region [3]. The full-length PRR14 protein consists of 585 amino acids and contains one proline-rich region [3]. The poline-region is related to signal transduction via binding with various domains [4-6]. A recent study found that PRR14 was able to directly bind with the growth factor receptor bound protein 2 (GRB2) through the proline-rich region mediated method and activate phosphatidyl Inositol 3-kinase (PI3K) pathway, consequently contributing to lung carcinogenesis [7]. The high-level mRNA expression of PRR14 was associated with worse 5-year survival overall in lung cancers [7]. However, the expression of PRR14 has not been assessed at the protein level ever before in lung cancers. Thus, we conducted the study to investigate the PRR14 protein expression in resected NSCLC patients using immunohistochemistry (IHC) and evaluate the associations between the PRR14 protein expression and prognosis of NSCLC patients.

Materials and methods

Patients and specimens

From 2008 to 2011, a total of 199 NSCLC patients who underwent complete resection of the primary tumor in West China Hospital were subsequently enrolled in our study. All patients received standard therapies after surgery according to the non-surgical management for lung cancer of Clinical Oncology Information

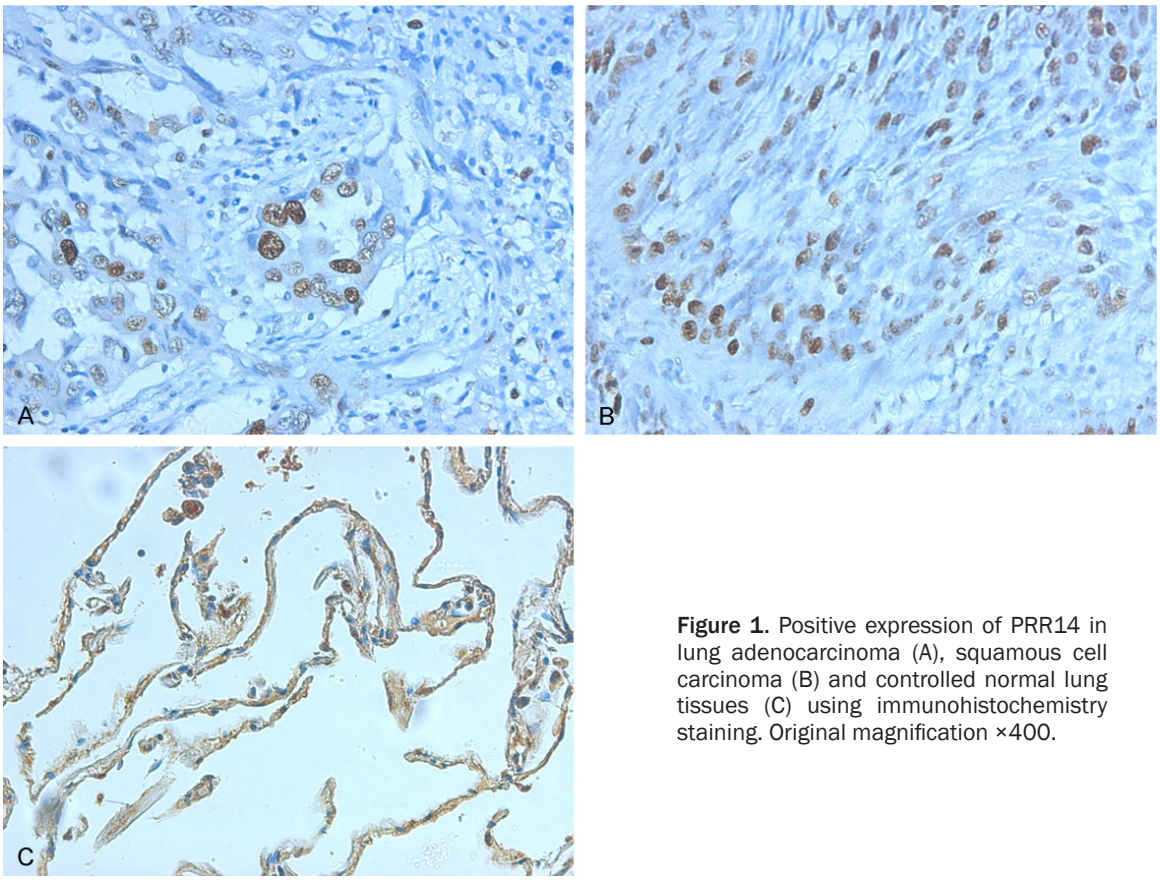

Figure 1. Positive expression of PRR14 in lung adenocarcinoma (A), squamous cell carcinoma (B) and controlled normal lung tissues (C) using immunohistochemistry staining. Original magnification ×400.

Network guidelines. Patients with previous malignancies, neoadjuvant therapies or incomplete clinical data were excluded. Tumor specimens and normal lung tissues adjacent to tumor were fixed in formalin and embedded in paraffin immediately after resection. Clinical data including gender, age, smoking status, histological type, differentiation, stage and lymph node infiltration were collected. Tumors were staged on the basis of the tumor-node-metastasis (TNM) staging system of the America Joint Committee on Cancer (AJCC 7th edition) [8]. Histological types were evaluated according to the World Health Organization classification for NSCLC [9].

All patients were followed up until cancer-related deaths or more than five years after surgery. Overall survival (OS) was defined as the period from the date of primary diagnosis to the date of cancer-related deaths or the end of follow-up. Progression-free survival (PFS) was defined as the time interval from the date of surgery to the date of first documented disease progression [10] or death. Written informed consents were obtained from all patients and our study gained the approval of the Committee on Medical Ethics of West China Hospital, Sichuan University.

Immunohistochemistry

Paraffin-embedded tissues were made into 4 μm-thick sections. The primary antibody was rabbit polyclonal IgG antibody purchased from Novus Corporation (NBP2-31812, Littleton, USA). Secondary antibodies were goat anti-rabbit antibodies purchased from Dako Corporation (Denmark). We conducted the immunohistochemistry staining according to the Envision two-step method [11].

First, Paraffin sections were dewaxed in xylene and dehydrated in the gradient ethanol series. Then the antigen retrieval was done in sodium citrate buffer (pH 6.0) at 95°C for 16 minutes. After cooling to room temperature, Endogenous peroxidase was blocked with 3% H_2O_2-methanol solution for 20 minutes. Sections were incubated with the 1:100 diluted primary antibodies at 4°C overnight. And then the secondary antibodies were incubated for 60 minutes at room temperature. The chromogen was 3,3'-diaminoben-

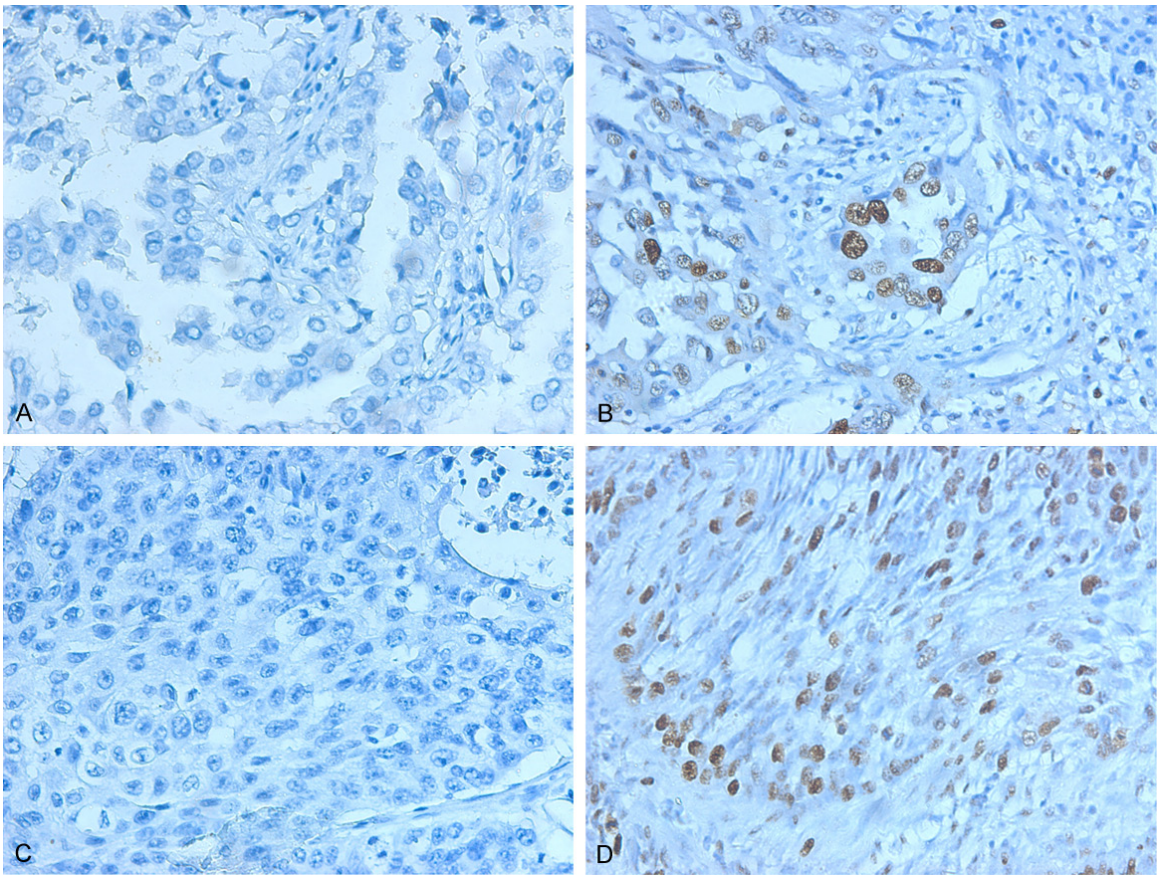

Figure 2. Expression of the PRR14 protein detected by immunohistochemistry in NSCLC samples. A. Negative, adenocarcinoma; B. Positive, adenocarcinoma; C. Negative, squamous cell carcinoma; D. Positive, squamous cell carcinoma; Original magnification ×400.

zidine (DAB). Finally tissues were counterstained with hematoxylin. The normal lung tissues were used as positive controls according to manufacturer's recommendations of the primary antibody. Negative controls, primary antibodies replaced by phosphate-buffered saline (PBS), showed no immunoreactivity.

Evaluation the expression of PRR14

Two pathologists blind to patient's clinical data carried out evaluations independently. PRR14 was nuclear positive in tumor samples. A total of three fields were randomly chosen to score for the areas of positively stained cells (brown) under light microscope. On average, more than 1% positively stained cells were defined as a positive expression.

Statistical analysis

Statistical analysis were conducted using SPSS 19.0 (SPSS, Chicago, USA) and graphs were made by Graphpad prism 6 (La Jolla, USA). Chi-square (χ^2) tests were employed to assess the association between clinicopathological features and PRR14 expression. The Kaplan-Meier curves and log-rank tests were used to evaluate survival. A multivariate Cox regression analysis was performed to estimate independent prognostic factors. *P* values ≤0.05 were considered statistically significant.

Results

PRR14 expression in lung tumor tissues and controlled normal samples

PRR14 was nuclear positively stained in some lung tumor tissues, while compared normal samples showed the membranous, cytoplasmic and rarely nuclear expression, as shown in **Figure 1**. PRR14 was positive in 95 out of 199 NSCLC samples (47.7%), and the remaining 104 specimens (52.3%) was negative expression. The expression of PRR14 protein in NSCLC patients was displayed in **Figure 2**.

Table 1. Clinicopathological features of the enrolled patients stratified by PRR14 expression

Variables	PRR14		*P* value
	Positive (n=95)	Negative (n=104)	
Age (year)			0.502
≤65	75	86	
>65	20	18	
Gender			0.002*
Male	74	59	
Female	21	45	
Smoking history			0.019*
Yes	64	53	
No	31	51	
Histological type			0.027*
Adenocarcinoma	46	62	
Squamous cell carcinoma	42	35	
Others	7	7	
Differentiation			0.980
Poor	50	54	
Moderate-well	45	50	
T stage			0.762
T1	12	11	
T2	60	68	
T3	16	14	
T4	7	11	
TNM stage			0.514
I-II	56	66	
III-IV	39	38	
Lymph node infiltration			0.050*
Yes	55	44	
No	40	60	

*Statistically significant.

PRR14 expression and clinicopathological characteristics

The relation among the expression of PRR14 and clinicopathological characteristics was demonstrated in **Table 1**. We found that PRR14 was significantly associated with gender, smoking history, histological type and lymph node infiltration in NSCLC patients (all P<0.05), while no statistical significance was observed between PRR14 expression and other clinicopathological variables.

PRR14 expression and survival

As shown in **Figure 3A**, about 54% NSCLC patients with PRR14 positive expression achieved the 5-year survival, significantly less than 74% for PRR14-negative patients (**Figure 3A**, P=0.002). The same trend was observed in lung adenocarcinoma (ADC) (**Figure 3B**) and lung squamous cell carcinoma (SCC) (**Figure 3C**) (all P≤0.05).

Furthermore, we found that the median progression-free survival (PFS) for the NSCLC patients with PRR14 positive expression was 15 months, significantly less than 30 months for PRR14-negative patients (**Figure 4A**) (P=0.006). The same trend was observed in lung ADC (**Figure 4B**) and SCC (**Figure 4C**) (all P≤0.05).

Clinicopathological variables, including age, gender, smoking history, lymph node infiltration and TNM stage were significantly related to OS in NSCLC patients (**Table 2**, all P<0.05). The patients' features including lymph node infiltration and TNM stage, were significantly related to PFS in NSCLC patients. Therefore, these variables were accordingly taken into multivariate Cox regression models.

More interestingly, we found that PRR14-negative NSCLC patients had more favorable OS (HR=0.522, 95% CI: 0.334-0.816) and PFS (HR=0.479, 95% CI: 0.286-0.803) through multivariate analysis (**Table 3**).

Discussion

For the first time, our study evaluated the expression of PRR14 in NSCLC tumor tissues using immunohistochemistry staining. In our study, the PRR14 protein was nuclear staining in lung tumor samples and positive expression in 95 out of 199 NSCLC patients (47.7%). PRR14 expression was significantly associated with gender, smoking history, histological type, lymph node infiltration in resected NSCLC patients (all P<0.05). Patients with PRR14-positive expression had worse 5-year survival (P=0.002) and shorter progression-free survival (PFS, P=0.006) than patients with PRR14-negative expression by univariate analysis. More interestingly, the multivariate analysis also suggested that PRR14 positive expression was significantly related to poorer OS and PFS, independent of the clinicopathological features of NSCLC patients.

Previous studies have found that the PRR14 protein was involved in nuclear localization sig-

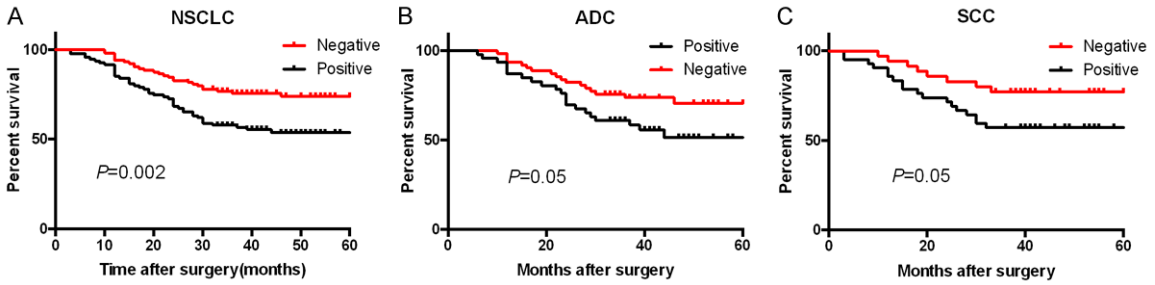

Figure 3. Kaplan-Meier survival curves showing the association between PRR14 expression and overall survival (OS) in non-small cell lung cancers (NSCLC) (A), lung adenocarcinomas (ADC) (B) and lung squamous-cell carcinomas (SCC) (C).

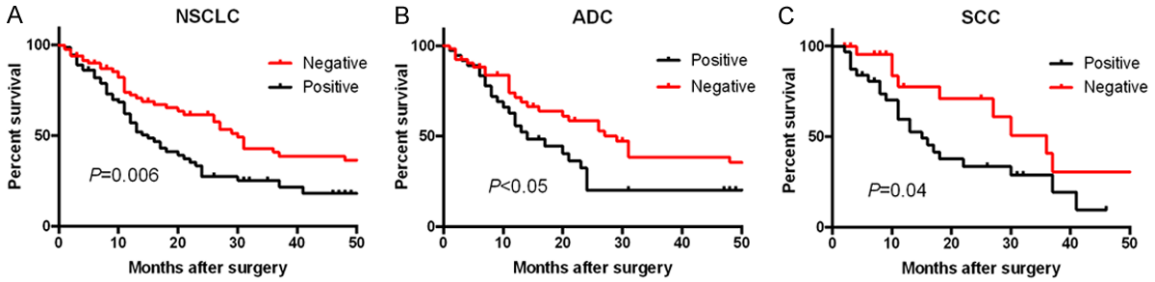

Figure 4. Kaplan-Meier survival curves showing the association between PRR14 expression and progression-free survival (PFS) in non-small cell lung cancers (NSCLC) (A), lung adenocarcinomas (ADC) (B) and lung squamous-cell carcinomas (SCC) (C).

Table 2. Univariate analysis about the survival of lung cancer patients

Variables	OS			PFS		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Age (>65/≤65)	2.035	1.307-4.632	0.006*	1.301	0.769-2.372	0.563
Gender (male/female)	1.855	1.073-2.869	0.026*	1.461	0.952-2.259	0.093
Smoking history (yes/no)	1.879	1.129-2.925	0.015*	1.289	0.852-1.987	0.236
Histological type (ADC/SCC)	1.039	0.633-1.709	0.878	1.000	0.641-1.560	1.000
Differentiation (poor/moderate-well)	1.031	0.643-1.654	0.899	1.305	0.862-1.999	0.213
Lymph node infiltration (yes/no)	2.634	1.635-4.331	<0.001*	2.933	1.969-4.913	<0.001*
TNM stage (III-IV/I-II)	2.634	1.635-4.331	<0.001*	2.699	2.015-4.914	<0.001*
PRR14 (positive/negative)	2.075	1.305-3.373	0.002*	1.754	1.203-2.826	0.006*

ADC, Adenocarcinoma; SCC, Squamous cell carcinoma; OS, Overall Survival; PFS, Progression-free Survival; *Statistically significant.

naling in human cells [3]. In mouse myoblasts, PRR14 was primarily localized in the nucleus and the surrounding of the nuclear membrane, with little distribution in the cytoplasm [12]. These are consistent with our results.

However, the relation among PRR14 expression and clinicopathological features in lung cancers has not been studied ever before. PRR14 has been identified as a member of the proline-rich family. Previous studies indicated that the proline-rich region was related to signaling via binding with various domains, particularly SH3 domains [4-6]. A recent study found that the proline-rich region of PRR14 is able to bind to the SH3 domain of GRB2, and consequently activates the PI3K/AKT/mTOR pathway in lung tumor cells. Multiple studies have found that the PI3K/AKT/mTOR pathway was frequently involved in the cell migration [13, 14], motility and invasion [15-17] in malignancies. Furthermore, other proteins containing the proline-rich

Table 3. Multivariate analysis about the survival of lung cancer patients

Variables	OS			PFS		
	HR	95% CI	*P* value	HR	95% CI	*P* value
PRR14 (positive/negative)	0.522	0.334-0.816	0.004*	0.479	0.286-0.803	0.005*

ADC, Adenocarcinoma; SCC, Squamous cell carcinoma; OS, Overall Survival; PFS, Progression-free Survival; *Statistically significant.

region were also found associated with tumor invasion. For example, the proline-rich tyrosine kinase2 (Pyk2) play a role in hepatocellular carcinoma progression. In their study, patients with Pyk2 overexpression had larger tumor size and advanced grading. Pyk2 overexpression was found in infiltrative tumor cells and lung metastatic nodules [18]. Further studies identified that overexpression of Pyk2 promoted invasiveness of hepatocellular carcinoma cells through up-regulating phosphorylation of c-Src at tyrosine residue 419 (Y419), and thus up-regulating c-Src activation [19]. Therefore, the proline-rich region mediated signaling may play an important role in the NSCLC metastasis processes, but more studies are needed to determine the mechanism between PRR14 expression and lymph node infiltration in NSCLCs.

The high-level mRNA expression of PRR14 was observed in multiple malignancies, including bladder urothelial carcinoma, cervical squamous cell carcinoma, colorectal and breast carcinoma [20]. However, there are few studies evaluated the expression of PRR14 and prognosis of carcinomas. A recent study found that the high-level mRNA expression of PRR14 associated with worse 5-year survival in human lung cancers [7], consistent with our results. Furthermore, they also demonstrated that PRR14 could activate the PI3K/AKT/mTOR pathway [7]. The PI3K/AKT/mTOR pathway has been found involved in the cell cycle progression [21-23], cell growth [24], proliferation [25], differentiation [26-28], survival [29], apoptosis [30, 31], angiogenesis [32], migration [13, 14], motility and invasion [15-17] in malignancies. The meta-analysis has confirmed that the PI3K/AKT/mTOR pathway activation is related to worse 5-year survival in solid tumors [33]. Therefore, the PRR14-mediated PI3K/AKT/mTOR pathway activation may contribute to tumor progression in NSCLC patients. Studies have found that the inhibition of PRR14 by siRNAs resulted in lower proliferation rate and lower colony formation capacity in human NSCLC cell lines. The PRR14 overexpression promoted lung cancer formation in mice. Nevertheless, more studies are needed to confirm the relation between PRR14 expression and prognosis of lung cancers.

In summary, PRR14 was related to tumor metastasis and indicated worse survival in resected NSCLC patients. Moreover, PRR14 was an independent predictor of unfavorable prognosis in resected NSCLC patients and may serve as a potential target.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Weimin Li, Department of Respiratory Medicine, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China. Tel: +86 28 85582944; Fax: +86 28 85582944; E-mail: weimi003@yahoo.com

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