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Original Article

Overexpression of immunohistochemical RhoA associated with poor prognosis in operable non-small cell lung cancer

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Abstract: Aim: The aim of the current study was to better understand the pathogenesis of non-small cell lung cancer (NSCLC). Research was conducted, investigating the relationship between RhoA expression and clinicopathological parameters of NSCLC. Lung cancer has the highest mortality rate of all cancers. Thus, survival curves were plotted, evaluating the prognostic value of RhoA. Material/methods: In 2005, a total of 140 tissue samples were acquired from patients with operable NSCLC (stage I-III). Immunohistochemical staining of RhoA was performed. Correlation levels between RhoA expression and clinicopathologic outcomes, as well as the prognostic value of RhoA, were analyzed. Results: RhoA showed a cytoplasmic pattern of expression in tumor tissues, while normal lung components showed negative or weak cytoplasmic staining. According to RhoA expression, the tumors were divided into low (n=74) and high (n=66) groups. High RhoA expression was associated with histology type in these patients (*P*=0.027). Compared to low RhoA expression, high RhoA expression showed significant differences with TNM stage (*P*=0.010) and development of lymph node metastasis (*P*=0.010). Results also showed that high RhoA expression was related to poor prognosis (*P*=0.008), rendering the same results in stage I patients (*P*=0.042). However, multivariate analysis revealed no statistical significance (*P*=0.083). Conclusion: Present data strongly suggests that immunohistochemical RhoA protein expression has a positive prognostic value. In the future, RhoA may serve as a prognostic marker in operable NSCLC patients.

Keywords: Non-small cell lung cancer, RhoA, prognostic factor, immunohistochemistry, tissue microarray

Introduction

Lung cancer is a common disease, seriously harming human health. In recent years, mortality and incidence rates of lung cancer have increased significantly [1]. NSCLC is the major histological form of lung cancer, remaining the principle cause of cancer-related deaths and accounting for more than one million deaths per year [2]. Due to a lack of effective diagnostic methods in the early stages of lung cancer, 5-year overall survival rates of lung cancer remain poor [3, 4]. The current study, therefore, researched molecular mechanisms involved in the development and progression of NSCLC.

Small guanosine triphosphatases (GTPases) Rho proteins belong to the Ras super family of low molecular weight GTPases, which cycle between an active GTP-bound state and an inactive GDP-bound state [5]. Three distinct families of proteins, guanine exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs), exert this regulatory cycle. When they are in the GTP-bound active form, Rho GTPases interact with and activate downstream effector proteins, thereby regulating cytoskeletal dynamics and stimulating a variety of biological processes, including cell division, survival, migration, and adhesion [6]. RhoA is one of the oldest Rho GTPases. It is involved in many cellular processes. Moreover, it has been regarded as a prominent regulatory factor in other functions as well, including regulation of cytoskele-

tal dynamics, transcription, cell cycle progression, and cell transformation [7].

Many studies have indicated that RhoA plays an important role in malignant transformation. For example, Takami Y reported that RhoA is related to malignant transformation and progression of colorectal cancer. Additionally, activation of RhoA has been associated with lymph node metastasis [8]. Overexpression of RhoA promotes the proliferation and migration of cervical cancer [9]. Furthermore, a recent study showed that loss of RhoA expression prevents proliferation and metastasis of SPCA1 lung cancer cells *in vitro* [10]. However, few studies have investigated the clinical significance of RhoA expression in NSCLC. The current study, therefore, set out to address these issues in a series of 140 patients with NSCLCs, evaluating expression of RhoA in relation to specific clinicopathological parameters and clinical information.

Materials and methods

Patients

In the current retrospective study, a total of 140 tissue samples of operable NSCLC (stage I-III) were obtained from patients (112 males and 28 females) that had undergone surgical resections in 2005. Complete clinical information was available for all patients. The average age of patients was 60 years (from 26 to 79 years). Patients were classified according to the tumor node metastasis (TNM) classification system, formulated jointly by the Union for International Cancer Control (UICC) and American Joint Committee on Cancer (AJCC). Selected patients had not received radiotherapy, chemotherapy, or biotherapy before surgery. The current study protocol was approved by the Research Ethics Committee of the Institutional Review Board.

Tissue microarray (TMA)

Samples of 140 patients were used. They were fixed and embedded by paraffin for construction of the TMA. Briefly, representative tumor areas were identified using paraffin blocks on corresponding hematoxylin and eosin (H&E)-stained sections, marking the areas of interest on the source block. A 1.0-mm-diameter precision punch (Beecher Instruments, Silver Spring, MD, USA) was used to core transfer the source block to the recipient block. To combat heterogeneity of the tumors, two 1.0 mm representative cores were selected after reviewing all original sections of the tumors. As described by Gao et al. [11], the tissues were arranged in rows and columns. A spreadsheet was elaborated to depict core locations. The TMA was cut into 4-μm sections and placed on glass slides. Manual Tissue Arrayer I (MTA-I; Beecher Instruments, Sun Prairie, WI, USA) was used in this study. Sections in the 30 slides were stained with H&E. An optical microscope was then used to ensure the presence of tumors and that all cores were present at the same depth. The slides were then used for immunohistochemistry (IHC) after drying for 16 hours at 60°C.

Immunohistochemical analysis

To detect expression levels of RhoA protein in NSCLC, standard indirect immunoperoxidase procedures (Envision Plus; Dako, Carpinteria, CA, USA) were used for immunohistochemistry. First, the xylene was dewaxed in the TMA section. It was then hydrated it in ethanol of different concentrations, according to the absolute concentration: 96%, 70%, and 40%. Each process lasted 5 minutes. Finally, it was washed with pure water. Next, endogenous peroxidase was quenched for 20 minutes with 0.3% hydroperoxidase. The sections were then subjected to heat-induced antigen retrieval in 10 mM citrate buffer for 10 minutes. Slides were incubated in 10 mM TBS with 4% normal goat serum for 1 hour and incubated with the primary anti-RhoA mouse monoclonal antibody (Abnova, Taiwan, China) at dilutions of 1:400. According to manufacturer instructions, biotin-free horseradish peroxidase enzyme-labeled polymer of the Envision plus detection system (Dako, Carpinteria, CA, USA) was used as a secondary antibody after washing with TBS. After exposing the sections to 3,3'-diaminobenzidine (Dako), the reaction products could be seen clearly. The sections were weakly counterstained with hematoxylin, dehydrated, and coverslipped. As reported previously [12], breast cancer tissue was used as a strongly positive control for RhoA. Phosphate buffer saline (PBS) was chosen instead of a pro-antibody as the negative control. Ensuring the accuracy of results, all TMA staining processes for each antibody were performed in a separate experiment.

Table 1. Clinical and histological features of 140 patients with NSCLC

Patient demographics	Number of of patients	Total (%)
Age[a]	60 (26-79)	
Sex		
Male	112	80
Female	28	20
Location		
Left	64	46
Right	76	54
Pathological type		
Squamous cell carcinoma	80	57
Adenocarcinoma	46	33
Adenosquamous cell carcinoma	5	4
Bronchiolo-alveolar carcinoma	3	2
Sarcomatoid carcinoma	1	1
Neuroendocrine carcinoma	3	2
Mucoepidermoid carcinoma	2	1
Differentiation		
Poorly	59	42
Moderate	49	35
Well	32	23
T stage		
1	15	11
2	105	75
3	20	14
N stage		
0	82	59
1	31	22
2	27	19
NSCLC stage		
I	67	48
II	44	31
III	29	21
Follow-up period[a]	45 (3-101)	

Notes: [a]Median (range).

Immunohistochemical evaluation

Immunohistochemical staining of RhoA showed brown granules in the cytoplasm. Based on a previous study of RhoA expression in ovarian carcinoma by Horiuchi A, cytoplasmic staining intensity was scored 0 to 3 in comparison to positive controls [13]. Tumors were considered negative when no staining or staining in <10% of neoplastic cells was observed. Weak staining (light yellow) in >10% of neoplastic cells was considered as 1+ positive. Moderate staining (yellow brown) in >10% of neoplastic cells indicated 2+ positive. Strong staining (brown) in >10% of neoplastic cells indicated 3+ positive. The patients were classified into either high expression (score 2 and 3) or low expression (score 0 and 1) groups. Two investigators assessed the evaluation of immunostaining, independently. When interpretations differed between observers, re-evaluations were conducted for a final decision using a conference microscope.

Statistical analysis

Correlation between RhoA expression and various clinicopathological parameters was determined by Pearson's Chi-square test. The period from primary surgery until the death of the patient or the latest follow-up is defined as overall survival (OS) time. None of the patients in this study died from a cause other than lung cancer. Univariate survival analysis of OS was performed, as outlined by the Kaplan-Meier method. This study used Cox's proportional hazards model to evaluate independent prognostic variables. *P* values <0.05 indicate statistical significance. Statistical analysis was performed using SPSS (version 19.0 for Windows, SPSS, Chicago, IL, USA). Results are expressed as the mean ± standard deviation (SD).

Results

Characteristics of the 140 patients

Table 1 shows an overview of the clinicopathological parameters of selected patients. The mean age was 60 years. More than half of the NSCLC cases were males. Of all patients, there were 80 cases of lung squamous cell carcinoma (LUSC, grade 1-3), 46 lung adenocarcinomas (LUAD, grade 1-3), 5 adenosquamous cell carcinomas, 3 large-cell neuroendocrine carcinomas, 3 bronchiolo-alveolar carcinomas, 2 mucoepidermoid carcinomas, and 1 sarcomatoid carcinoma. Clinical follow-ups were recorded until July 2013. There were 84 local recurrences or distant metastases and 91 deaths at the end of follow-up.

Immunohistochemical expression of RhoA

RhoA showed a negative or weak cytoplasmic staining in control cores, compared to normal lung tissues. In tumor cells, RhoA staining was detected mainly in the cytoplasm. Varying degrees of cytoplasmic staining were observed,

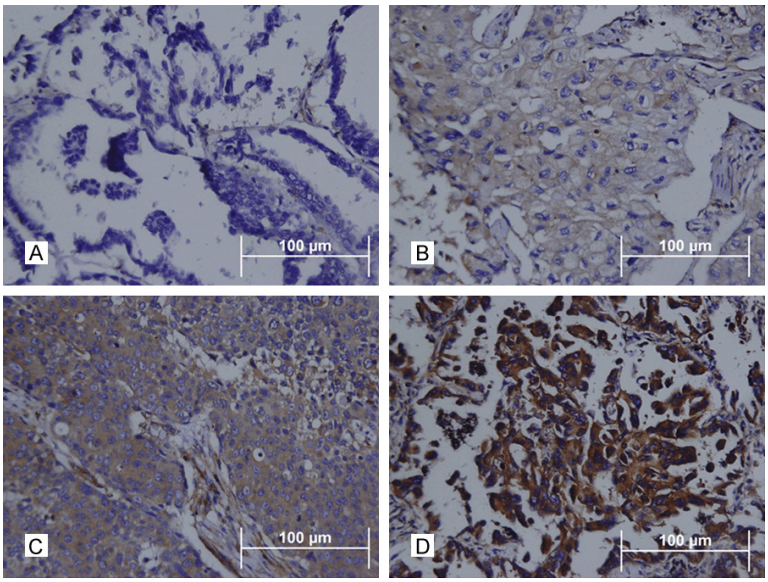

Figure 1. Immunohistochemical staining for RhoA in operable NSCLC. Tumor cell negative for RhoA (A); Displaying weak cytoplasmic staining of RhoA with an intensity of 1+ (B); An example of RhoA for expression with a moderate intensity of 2+ (C); Strong RhoA staining with an intensity with an intensity of 3+ (D).

including yellow or brown granules (**Figure 1**). Some cells, morphologically identifying as macrophages, showed abundant cytoplasmic RhoA immunoreactivity. These were excluded from the evaluation of RhoA staining in tumor cells. In total, RhoA reactivity was absent in 18 cases (13%, score 0), weak in 56 cases (40%, score 1+), moderate in 47 cases (34%, score 2+), and strong in 19 cases (14%, score 3+). The tumors were divided into low (n=74) and high (n=66) groups, according to intensity and extent of staining.

RhoA expression and clinicopathological parameters

RhoA expression was subdivided into low and high-expression groups. The current study compared clinicopathological characteristics between patients with low and high expression of RhoA. **Table 2** shows the association between RhoA expression and clinicopathological variables. High-expression RhoA promoted progression of N stage (P=0.010) and TNM stage (P=0.010). Significant correlation was found between RhoA expression and pathological type (P=0.027). Squamous cell carcinoma showed a higher level of RhoA expresion than non-squamous cell carcinoma. Howerver, there was no correlation with the other parameters.

RhoA expression and patient overall survival

Figure 2 shows Kaplan-Meier survival curves plotted for RhoA expression. Patients with low RhoA expression had higher 5-year overall survival rates (48.4 VS. 28.8%, P=0.008) than those with high RhoA expression. Statistically significant survival differences were shown between low and high levels of RhoA expression (P=0.042), according to survival analysis of patients with Stage I NSCLC. In this group, patients with high RhoA expression had lower 5-year overall survival rates (34.8% VS. 56.4%), compared to patients with low RhoA expression. Multivariate regression analysis details concerning potential factors influencing OS are shown in **Table 3**. Results suggest that only lymph node status (P=0.038) and age (P=0.005) had independent prognostic value.

Discussion

Lung cancer ranks first among lethal cancers. Moreover, NSCLC accounts for 80% of lung cancers [14]. Invasion and metastasis of tumors are the most important factors affecting prognosis. They involve changes of various cytokines, adhesion molecules, and matrix proteases. Recent studies have shown that Ras signaling pathways are involved in occurrence and development of cancer cells via promoting cell proliferation and migration [15]. Additionally, RhoA protein belongs to the Ras super family of low molecular weight GTPases. It is upregulated in a variety of human tumor types. It stimulates cell cycle progression and cytokinesis by regulating a diverse range of cellular functions, primarily through their ability to modulate microtubule dynamics and the actin-myosin cytoskeleton, as well as regulating cell migration [16-19].

Previous studies have indicated pro-oncogenic roles for RhoA protein in tumor progression. For

Table 2. Relationship between RhoA and clinicopathological factors

	RhoA		
	Low	High	*P*-value
Age	60.4±10.1	59.8±10.2	0.712
Sex			
Male	60	52	0.833
Female	14	14	
Location			
Left	32	32	0.611
Right	42	34	
Histological type[a]			
Squamous	49	31	0.027*
Non-squamous	25	35	
Differentiation			
Poorly	30	29	
Moderate	27	22	0.907
Well	17	15	
pT			
1	6	9	
2	56	49	0.493
3	12	8	
pN			
0	52	30	
1	13	18	0.010*
2	9	18	
TNM stage			
I	44	23	
II	20	24	0.010*
III	10	19	

Notes: [a]Some tumors, such as adenosquamous, bronchioloalveolar carcinomas, sarcomatoid carcinomas, neuroendocrine carcinomas, and mucoepidermoid carcinomas, were included in non-squamous cancer data. *Significant correlation.

example, high RhoA expression in esophageal carcinoma could promote proliferation and cell invasion of human esophageal cancer cells [20]. Furthermore, RhoA has been associated with invasion of lymph nodes and blood vessels in colorectal cancer samples. Patients with higher RhoA expression have a significantly poorer 5-year survival rate after surgery [21]. Moreover, RhoA knockdown has been shown to prevent cell proliferation and induces apoptosis in SPCA1 lung cancer cells [22]. RhoA expression is regulated by a number of microRNAs in tumors. Lei reported that miR-182 directly targets MIM (missing in metastasis), which suppresses metastasis by inhibiting RhoA activity and stress fiber formation in breast cancer cells [23]. These findings lead to further acknowledgement that RhoA regulates a diverse range of cellular functions, primarily through their ability to modulate microtubule dynamics and the actin-myosin cytoskeleton. Not surprisingly, RhoA is crucial for cell migration. It is, therefore, highly important for cancer cell invasion and the formation of metastases [24].

RhoA is critically involved in multiple stages of the tumorigenic process, raising the possibility that RhoA may be a useful prognostic indicator. Occurrence and development roles of RhoA in NSCLC have also been reported [25-28]. However, few studies have established its role in determining the prognosis in NSCLC. The current study examined RhoA expression in patients with operable NSCLC, comparing it to clinicopathologic parameters and overall survival using immunohistochemistry in TMA. Results showed that high expression of RhoA was associated with positive N-stage, pathological type, and correlated with TNM stage, while no association was observed between RhoA expression and any other clinicopathologic parameters. Consistented with these resluts, Huang also found that patients with high RhoA expression were associated with more advanced pathological N category than those with low RhoA expression in gastric cancer [29]. Faried et al. found that increased expression of RhoA was related to higher TNM stages of esophageal squamous cell carcinoma [30]. In the current study, patients with high expression of RhoA were found to have a poorer prognosis for 5-year survival than those with low RhoA protein expression. The same results were found in stage I patients. However, according to multivariate analysis, RhoA did not appear to be an independent prognostic factor. Lymph node metastasis maintained its independence for prognosis. Considering previous results suggesting that RhoA protein expression is closely related to lymph node metastasis, it was speculated that expression of RhoA in NSCLC promotes tumor invasion and lymph node metastasis, affecting prognosis of the disease.

In conclusion, present data strongly suggests that immunohistochemical RhoA protein expression has positive prognostic value. Overexpression of RhoA was found to be associat-

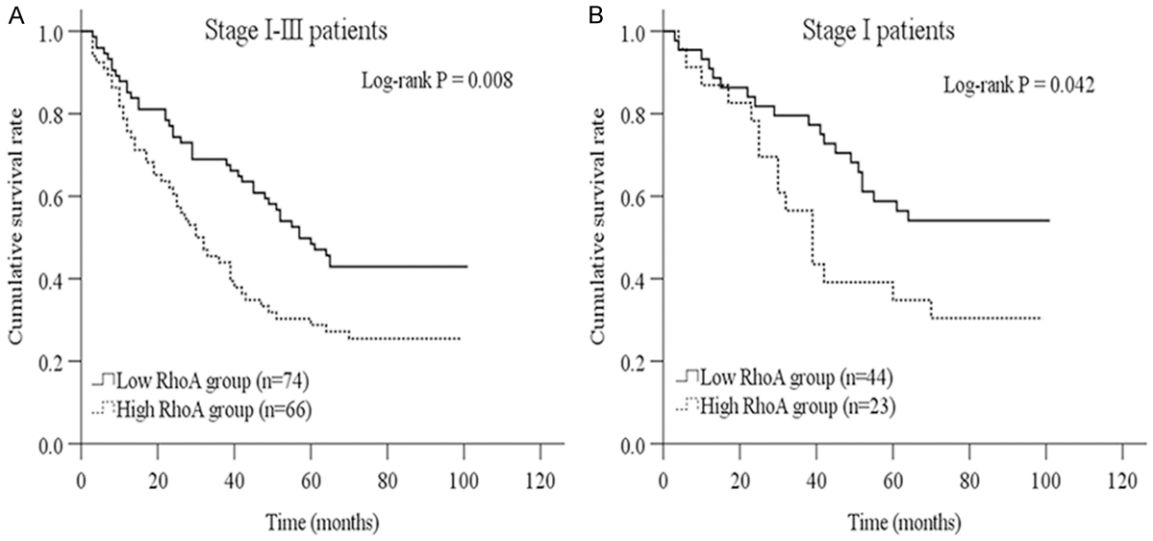

Figure 2. Kaplan-Meier survival estimates for operable NSCLC patients. Univariate survival analysis indicated that high RhoA expression is associated with poorer prognosis in patients with Stage I-III NSCLC (*P*=0.008, log-rank test) (A), as well as in Stage I patients (*P*=0.042, log-rank test) (B).

Table 3. Multivariate Cox regression analysis for potential factors influencing overall survival

Parameters	B	SE	Wald	*P*-value	OR	95% CI for OR	
						Lower	Upper
Univariate analysis							
Age	0.025	0.011	5.274	0.022*	1.026	1.004	1.048
Sex	-0.240	0.275	0.762	0.383	0.786	0.458	1.349
Location	-0.216	0.210	1.059	0.303	0.806	0.534	1.216
Histologicaltype	-0.123	0.213	0.337	0.562	0.884	0.583	1.341
Differentiation	-0.014	0.139	0.010	0.922	0.987	0.752	1.295
pT	0.839	0.423	3.940	0.047*	2.314	1.011	5.298
pN	0.825	0.212	15.166	0.000*	2.281	1.506	3.454
Stage	0.655	0.215	9.275	0.002*	1.926	1.263	2.937
RhoA	0.549	0.211	6.751	0.009*	1.732	1.144	2.621
Multivariate analysis							
Age	0.022	0.011	4.300	0.038*	1.022	1.001	1.044
pT	0.740	0.428	2.990	0.084	2.095	0.906	4.845
pN	0.636	0.225	7.976	0.005*	1.888	1.215	2.935
RhoA	0.386	0.223	3.001	0.083	1.472	0.951	2.278

B: Regression coefficient; SE: standard error; Wald: Wald test; OR: odds rario; CI: confidence interval. *Significant correlation.

ed with poor prognosis. Thus, it may serve as a prognostic biomarker in operable NSCLC.

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Disclosure of conflict of interest

None.

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