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Original Article

Expression of Raf kinase inhibitor protein and radiotherapy prognosis of non-small-cell lung cancer

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Abstract: *Background:* Raf kinase inhibitor protein (RKIP) is thought to be an inhibitor of multiple cellular signaling pathways and a suppressor of cancer metastasis in a variety of human cancers. However, function of RKIP in non-small cell lung cancer (NSCLC) is not yet completely understood. The purpose of this study was to investigate the relationship between the RKIP expression in tumor tissues of NSCLC and the prognosis of NSCLC patient following radiotherapy (RT). *Methods:* The level of RKIP expression in tumor tissue samples from 77 NSCLC patients was retrospectively determined by using immunohistochemistry. Survival rate was estimated by Kaplan-Meier curves. Log-rank univariate analysis was used to identify the factors with prognostic significance and the results were verified by multivariate Cox proportionate hazard regression analysis. *Results:* Among the 77 NSCLC patients, 62 (80.5%) patients had low RKIP expression and 15 (19.5%) had high expression. The 1-, 2- and 3- year survival rates were 62.3% (48/77), 40.3% (24/77) and 27.3% (4/77), respectively. The Cox regression model showed that patients in low RKIP expression group had a significantly higher risk of death (RR = 2.141) than those in high RKIP expression group ($P < 0.05$). Multivariate analyses revealed RKIP expression was an independent factor that affected overall survival ($P < 0.05$). *Conclusion:* RKIP could be a potential biomarker for the prognosis of NSCLC patients after RT.

Keywords: Non-small cell lung cancer, Raf kinase inhibitor protein, prognosis, radiotherapy, immunohistochemistry

Introduction

Raf kinase inhibitor protein (RKIP) is a member of phosphatidylethanolamine-binding protein (PEBP) family. The most thoroughly explored biological role of RKIP is that RKIP mediated a number of intracellular signaling pathways. RKIP inhibits the signaling pathway of Raf-1-MEK1/2-ERK1/2 and restrain the signal conduction process of G protein-coupled receptor kinase [1-3]. Its activity is regulated by phosphorylation [4]. Expressions of RKIP in some cancer tissues, such as breast cancer, prostate cancer, melanoma, lung squamous cell carcinoma, esophageal, carcinoma of rectum and liver cancer, are lower than those in normal tissues [5-8]. Some scholars believe that RKIP expression may improve the radiosensitivity of nasopharyngeal cells [9], but no studies to date have investigated whether RKIP expression correlates with the prognosis of non-small cell lung cancer (NSCLC) following radiotherapy (RT).

RT is the main therapy for NSCLC patients who are not suitable candidates for surgery [10]. However, the overall survival rate of NSCLC over 5 years is lower than 15% [11] and the 5-year overall survival after RT was about 10% [12]. The main reasons for this low survival rate include uncontrolled growth of the primary tumor, recurrence and metastasis; the rate of occurrence of uncontrollable primary tumor growth is 70% [13]. Radiation resistance is the principal biological factor that influences the efficacy of RT [14]. Therefore, looking for an indicator of RT outcome is the key to achieve individualized and efficient treatments for patients with NSCLC. For this reason, the search for biomarkers of radiation efficacy has been a pressing concern in clinical radiation oncology [15].

This study investigated RKIP expression levels in NSCLC pathological specimens and followed up the patients after RT, aiming to discover an independent prognosticator, in order to provide

Table 1. Distribution of general materials and Comparability Test of 77 NSCLC Patients Receiving Radiotherapy

Factor		Low RKIP	High RKIP	χ^2	*P* value
Gender					
	Male	13	47	0.317	0.573
	Female	2	15		
Age					
	≤ 60	8	32	1.896	0.905
	> 60	7	30		
Smoking status					
	Yes	3	21	0.533	0.465
	No	12	41		
KPS					
	≤ 80	2	13	0.094	0.759
	> 80	13	49		
Pathological type	SC	11	42	0.012	0.913
	AC	4	20		
Clinical stage	Stage II	3	17	0.068	0.795
	Stage III	12	45		

NSCLC: non-small cell lung cancer; RKIP: Raf Kinase inhibitor protein; KPS, Karnofsky Performance Status; SC: squamous cell carcinoma; AC: Adenocarcinoma.

useful information for the correct evaluation of the patients' prognosis, with the ultimate goal of providing suitable individualized treatment.

Materials and methods

Patients and specimens

This study was subject to approval by the Research Ethics Committee of the First Affiliated Hospital of China Medical University, China.

NSCLC patients whose karnofsky performance status (KPS) were all above 70 and who were accepted three-dimensional (3D) conformal RT between Jan 2007 and Dec 2010 at the Department of Radiotherapy, First Affiliated Hospital of China Medical University, were included in this study. Patients were excluded from the study if they had had a break from RT for more than five days, radiation with inconsistent doses and a total radiation dose < 50 Gy. Thus, a total of 77 patients enrolled in this study and they were prospectively followed up after the RT was completed. The clinical data of patients were retrospectively reviewed by authors. The NSCLC tissue samples those were embedded in paraffin were used for reappraising of the pathological type (squamous cell carcinoma or adenocarcinoma) and clinical stage by two pathologists according to the AJCC lung cancer TNM stage in 2009.

Immunohistochemistry analysis

Expression of RKIP was investigated by immunohistochemistry according to the previous study [16]. The sections (4 μm) were dewaxed in a graded ethanol series. Subsequently, the activity of endogenous peroxidase was suppressed through incubating the sections with 0.3% hydrogen peroxidase and methanol for 20 min at room temperature. After washed in phosphate buffered saline (PBS), the slides were incubated with the rabbit monoclonal anti-RKIP antibody (Santa Cruz, CA, USA) overnight at 4°C. Then the secondary anti-rabbit IgG antibody (Sigma, St. Louis, MO, USA) were added. The sections were developed with a DAB (diaminobenzidine) detection kit.

Independent scoring of immunohigtochistry was conducted according to the study of Yu *et al.* [17] by two pathologists who were blinded design of this study. Any disagreement was dissolved by reexamination. RKIP protein was found to be predominantly cytoplasmic, although some nuclear staining was noted. The criteria for the scoring were as follow: the total score was the product of percentage of positive cells and the intensity of staining. Samples with total score > 3 scores were identified as high RKIP expression, while those ≤ 3 scores were identified as low RKIP expression.

Radiation treatment and follow-up

The procedures of 3D conformal RT was similar with those described in previous study [18]. Briefly, localization of the gross tumor volume (GTV) was the total volume of the primary and nodal tumor masses determined by computed tomography (CT) scans, while the clinical target volume (CTV) was obtained by the addition of a 0.6 cm × 0.8 cm margin to GTV. The planning target volume (PTV) equals to the volume that a 0.5 cm × 1.0 cm edge plus to the outside of CTV. The prescribed dose per fraction for patients received only RT was 62.5-65 Gy, and those for patients who underwent concurrent

Table 2. Distribution of clinical materials

Factors	Cases	Drugs for chemotherapy
Initial treatment without surgery	64	
Postoperative recurrence	13	
Only radiotherapy	28	
Radiotherapy and chemotherapy	49	
Sequential chemotherapy and radiotherapy	21	DP or NP
Concurrent chemotherapy and radiotherapy	20	EP or NP
Consolidation chemotherapy ≥ 2 cycles	11	DP or NP

DP for Docetaxel and cisplatin, docetaxel (75 mg/m^2 on day 1) and cisplatin (20-25 mg/m^2 on days 1-3); NP for Vinorelbine and cisplatin, vinorelbine (20 mg/m^2 on day 1 and 5) and cisplatin (20 mg/m^2 on days 1-3); EP for Etoposide and cisplatin, etoposide (100 mg/m^2 on days 1-5) and cisplatin (20-25 mg/m^2 on days 1-3).

Table 3. Causes of death

Causes of death	Cases	Percentage
Uncontrolled growth of the primary tumor	16	26.70%
Distant metastasis	13	21.70%
Uncontrolled growth of the primary tumor & distant metastasis	28	46.70%
Interstitial pneumonia or lung fibrosis	1	1.70%
Unrelated to NSCLC	2	3.30%

NSCLC: non-small cell lung cancer.

Table 4. The median survival time

Groups	Median survival time (months)
Radiation dose > 60 Gy	34.2
Radiation dose ≤ 60 Gy	14.3
RKIP low expression	9.5
RKIP high expression	20.0

RKIP: Raf Kinase inhibitor protein.

or sequential chemoradiotherapy was 2.0-2.5 Gy with a total dosage of 60-62.5 Gy. The treatment plan usually consisted of five or six RT sessions in one week in four or five fields.

All patients were followed up by their treating radiation oncologist monthly in the first three months after RT and then every three months until the deadline December 2012.

Statistical analysis

SPSS 19.0 (Chicago IL, USA) and SAS 9.1 (Cary, NC, USA) were used for statistical analyses. The associations of RKIP expression to the clinical factors was evaluated by χ^2 test. The overall survival of NSCLC patients were estimated by Kaplan-Meier method and compared using log-rank univariate test. The results of univariate analysis were further verified by a multivariate Cox regression analysis. $P < 0.05$ was used as the threshold of significance.

Results

Immunohistochemical findings in RKIP

RKIP expression was mainly in the NSCLC tissue cytolymph, which are often yellow granules. Among the 77 NSCLC tissues, the number of samples with low RKIP expression was 15 (19.5%) and it was 62 (80.5%) with high RKIP expression (**Table 1**). There were no significant differences in gender, age, smoking status, KPS, pathological type, clinical stage between low RKIP group and high RKIP group ($P > 0.05$). **Table 2** showed the distribution of clinical treatment for the patients. Most of the patients were initial treatment without surgery (64/77), and 28 of 77 patients received only radiotherapy.

Survival time of NSCLS patients after RT

At the last follow-up date in December 2012, 60 patients (77.92%) had died. The median follow-up was 17.5 months (3-52 months). Three cases were lost. After RT, the 1-, 2- and 3-year survival rates were 62.3% (48/77), 40.3% (24/77) and 27.3% (4/77), respectively. The overall median survival time was 17.2 months. Causes of death are shown in **Table 3**, The prominent cause is uncontrolled growth of the primary tumor & distant metastasis (46.70%). **Table 4** shows that patients accepted high dose radiation (> 60 Gy) and those with high RKIP expression survived longer than those with low radiation dose and low RKIP expression.

Single factor analysis

The results above were further verified by Log-rank single factor analysis (**Table 5**). The findings suggested that several factors such as

Table 5. The Log-rank analysis of 77 NSCLC Patients after radiotherapy

Factor	Cases	Survival rate (%)			χ^2	*P* value
		1 year	2 year	3 year		
Gender						
Male	60	61.7	40.0	30.0	0.082	0.774
Female	17	64.7	41.2	17.6		
Age						
≤ 60 year	40	55.0	27.5	20.0	3.426	0.064
> 60 year	37	70.3	54.1	35.1		
Smoke						
No	53	69.8	47.2	35.8	5.205	0.023
Yes	24	45.8	25.0	8.3		
KPS						
≤ 80	15	60.0	33.3	0.0	0.696	0.404
> 80	62	62.9	41.9	33.9		
Pathological type						
SC	53	58.5	45.3	30.2	0.418	0.518
AC	24	70.8	29.2	20.8		
Clinical stage						
Stage II	20	85.0	65.0	35.0	5.447	0.02
Stage III	57	54.4	31.6	24.6		
RKIP						
Low	15	46.7	26.7	6.7	5.815	0.016
High	62	66.1	43.5	32.3		
T state						
T_{1-2}	56	73.2	50.0	35.7	18.886	< 0.001
T_{3-4}	21	33.3	14.3	4.8		
N state						
N_1	23	82.6	56.5	30.4	8.495	0.014
N_2	39	59.0	41.0	30.8		
N_3	15	40.0	13.3	13.3		
Radiation dose						
≤ 60 Gy	50	54.0	26.0	18.0	14.67	< 0.001
> 60 Gy	27	77.8	66.7	44.4		
Chemotherapy*						
Yes	49	55.1	28.6	22.4	5.669	0.017
No	28	75.0	60.7	35.7		
Operation						
Yes	13	61.5	53.8	38.5	0.282	0.595
No	64	62.5	37.5	25.0		

*Chemotherapy more than 2 cycle; RKIP: Raf Kinase inhibitor protein; SC: squamous cell carcinoma; AC: Adenocarcinoma.

clinical stage, smoking status, RKIP expression, clinical staging, staging T, staging N, RT dose and chemotherapy were correlated with the prognosis of NSCLC after RT ($P < 0.05$), while the pathological type, surgical treatment, KPS, age and gender had no correlation with the prognosis.

Multiple factor analysis

Cox analyses (**Table 6**) showed that smoking status, RKIP expression and RT dose were independent prognosis factors ($P < 0.05$). Survival graphs for smoking status, RKIP expression and RT dose were shown as stratified factors (**Figure 1**).

Discussion

Downregulation of RKIP expression in patients with NSCLC correlates with poorer differentiation and advanced pathologic TNM stage [19]. RKIP is a metastasis suppressor for a number of cancers [20]. Recent reports have confirmed that RKIP can inhibit Raf-1/MEK/ERK pathway [21] and involve in the adjustment of the NF-KB signal transduction pathway [22]. The abnormal activation of these signaling pathways is closely related to the development of tumors [4, 23, 24]. RKIP expression in various tumor tissues is lower than in normal tissue [7, 8]. Downregulation of RKIP expression is related to metastasis and poorer prognosis in colorectal cancer, pancreatic cancer and other malignancies [25, 26]. In this study, we found RKIP is an independent prognostic factor of NSCLC patients following RT.

After RT, the survival rate of NSCLC patients with high level of RKIP expression is higher than those with low RKIP, suggesting that RKIP expression level predicts the outcomes of NSCLC patients following RT. However, Houben *et al.* reported that the RKIP was expressed homogeneously in virtually all melanoma samples, and the level of RKIP expression had no relation with primary melanoma patients' 5-year survival rate [27]. The discrepancy in finding could be caused by the different expression state of RKIP in different tumor, so more study is needed.

Xu *et al.* reported [28] the relationship between RKIP and the prognosis of hepatocellular carci-

Table 6. Multivariate Cox regression analysis for 77 NSCLC Patients survival after radiotherapy

Factors	β	SE	χ^2	*P* Value	OR	95% CI	
						Lower	Upper
RKIP expression	0.761	0.349	4.770	0.029	2.141	1.081	4.240
Total Dose	1.110	0.376	8.724	0.003	3.033	1.452	6.333
Smoke	-0.979	0.305	10.297	0.001	.376	0.207	.683
Clinical stage	-0.544	0.373	2.126	0.145	.581	0.280	1.206
Chemotherapy*	0.560	0.336	2.778	0.096	1.751	0.906	3.384

RKIP: Raf Kinase inhibitor protein; *Chemotherapy more than 2 cycle.

noma (HCC) patients after surgery. Their result shows that expression of RKIP in HCC patients is low and that the level of expression correlates with the invasiveness of the tumor. Thus, they concluded that RKIP may be a predictor of post-operative recurrence for HCC patients. They further suggested that decreased RKIP expression levels may lead to changes in the MAPK signal conduction pathway, thereby causing cell apoptosis. However, the exact role of RKIP in apoptosis pathways requires further study.

RKIP is able to improve the sensitivity of a variety of malignant cells to the presence of anticancer drugs [29-31]. Chemotherapeutic agents could rapidly induce RKIP expression by reducing the signal of NF-KB and RAF1, thereby inducing tumor cell apoptosis [32]. Recently, Cross *et al*. have indicated that phosphorylation of RKIP is related to the effects of chemotherapeutics and the prognosis of colon cancer [33]. However, we do not yet understand how an increase of RKIP expression could lead to an increase of the sensitivity of tumors to chemotherapeutic agents.

It is important to emphasize that some researchers [34] have experimentally manipulated the level of RKIP expression in C4-2B PCa prostate cancer cells. By transplanting the recombinant prostate cancer cells into mice and irradiating, the authors found that the cells in which RKIP expression was increased showed increased radiosensitivity. They speculated this may be caused by cell apoptosis through poly adenosine diphosphate ribose polymerase (PARP) cleavage. Other scholars [9] did a similar study using a nasopharyngeal carcinoma (NPC) cell line, and the result illustrates that reducing RKIP expression in the NPC cells can improve the resistance of cells to cell cycle arrest, death or apoptosis during RT, which is related to the regulation of phosphorylation in signal pathways MEK and Raf-1/MEK/ERK, thereby regulating the radiosensitivity of NPC cells. These basic research results mentioned above suggest that the absence of RKIP influences the efficacy of RT for malignant tumors by increasing radiation resistance of tumor cell.

Exploring the influence of RKIP expression in NSCLC RT prognosis may lead to changes in aspects of treatment strategies for patients with NSCLC. This will lead to development of improved, individualized and efficient RT therapies for NSCLC patients, while, also providing the theoretical basis for understanding changes in radiosensitivity to specific markers in NSCLC. Nevertheless, the specific mechanisms need further study.

Conclusion

The present study indicates that smoking, RKIP expression, clinical stage, T stage, N stage and radiation dose are related to RT prognosis in NSCLC, while pathological type, surgical treatment, KPS, age and gender have no relationship to the RT prognosis of NSCLC. Importantly, smoking, RKIP expression and RT dose are independent prognostic factors in NSCLC patients, however the exact mechanisms need more study.

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Disclosure of conflict of interest

None.

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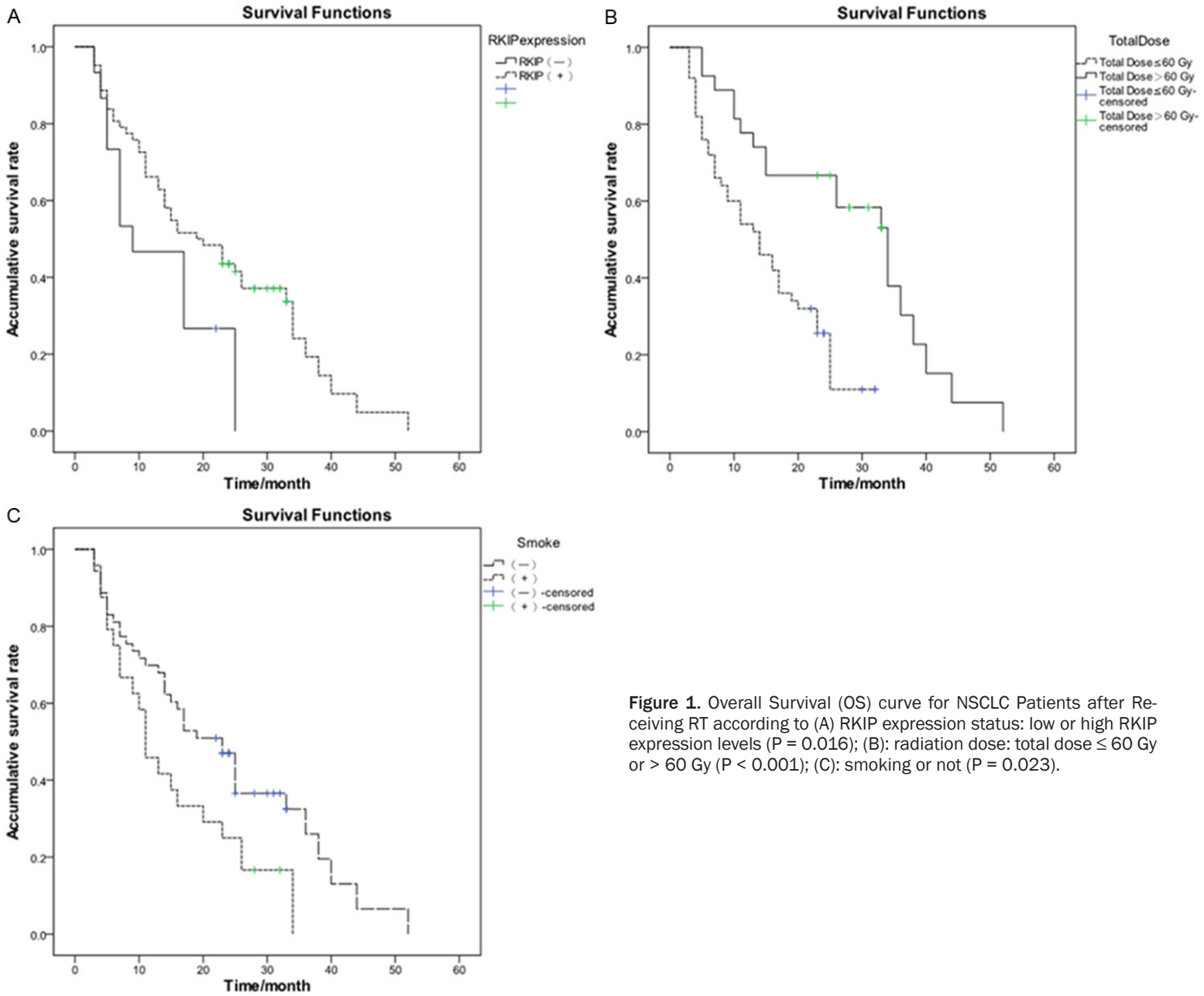

Figure 1. Overall Survival (OS) curve for NSCLC Patients after Receiving RT according to (A) RKIP expression status: low or high RKIP expression levels (P = 0.016); (B): radiation dose: total dose ≤ 60 Gy or > 60 Gy (P < 0.001); (C): smoking or not (P = 0.023).

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