RESEARCH ARTICLE

Prognostic Value of Phosphorylated mTOR/RPS6KB1 in Non-small Cell Lung Cancer

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Abstract

Background: The mammalian target of rapamycin (mTOR) /RPS6KB1 activation has recently been implicated in tumour development, but its role in lung cancer remains unclear. The aim of this study was to explore the role of mTOR/RPS6KB1 signaling pathway in non-small-cell lung cancer (NSCLC). Methods: Immunohistochemistry was performed to assess the expression of phosphorylated mammalian target of rapamycin (p-mTOR) and its downstream ribosomal phosphorylated RPS6KB1 (p-RPS6KB1) in NSCLC patients. We also analyzed p-mTOR/ p-RPS6KB1 protein expression in 45 fresh NSCLC tissues using Western blotting. Results: The expression level of p-mTOR and p-RPS6KB1 was significantly higher in NSCLC tumor specimens than that in adjacent noncancerous normal lung tissues ($P<0.01$). p-mTOR expression correlated with p-RPS6KB1. Furthermore, high expression level of p-mTOR or p-RPS6KB1 in NSCLC was associated with a shorter overall survival (both P<0.01). Multivariate analysis indicated high level of p-mTOR expression was an independent prognostic factor (HR=2.642, 95%CI 1.157–4.904, $p=0.002$). Conclusions: p-mTOR and p-RPS6KB1 could be useful prognostic markers for NSCLC.

Keywords: Non-small-cell lung cancer - biomarker - mTOR/RPS6KB1 - prognosis

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Introduction

Lung cancer is one of the most common and lethal cancers all over the world (Malik et al., 2013). Non-small cell lung cancer (NSCLC) accounts for approximately 75% of all cases of lung cancer (Minna et al., 2002). Identification of potential molecular targets of NSCLC is critically important for developing molecular-profile-directed therapy, which appears promising in early clinical studies (Thomas et al., 2013). However, the prognosis of these patients remains poor as the 5-year overall survival is less than 15% (Jemal et al., 2010). These unsatisfactory outcomes highlight the need for novel reliable predictors of survival and novel therapeutic targets.

The mammalian target of rapamycin (mTOR) is a protein kinase of the PI3K/Akt signaling pathway and plays a crucial role in the control of cell proliferation, survival, mobility and angiogenesis (Ferrara et al., 2003). Dysregulation of mTOR pathway has been found in numerous human cancers (Wang et al., 2011). The activation of mTOR and its downstream protein RPS6KB1 play a key role in carcinogenesis (Chen et al., 2002). Some studies suggest that rapamycin and its analogs can act as inhibitors of mTOR, capable of inhibiting the proliferation and growth of pancreatic endocrine tumors (Menon et al., 2008). However, the association of mTOR signaling pathway with NSCLC remains unclear.

To evaluate the role of mTOR/RPS6KB1 signaling pathway in NSCLC, we collected NSCLC samples in order to investigate the association of mTOR/ RPS6KB1 activation with the survival of NSCLC using immunohistochemical staining.

Materials and Methods

Patients and tissue samples

NSCLC (n=120) tissues and adjacent noncancerous normal lung tissues (n=78) were obtained from January 1st 2003 to January 1st 2011, following surgical resection at our Hospital. None of the patients had received radiation therapy or chemotherapy before surgery. Clinicopathological features are shown in Table 1. The patients had a median age of 61 years (range 24-83 years). Sections of tumor were reviewed by two independent pathologists to determine the histological type. The TNM staging system of the International Union Against Cancer (7th Edition) was used to classify specimens as stages I (n=38), II (n=34), III (n=40), and IV (n=8). The 120 cases had complete follow-up records. The survival time was calculated from the operation to death or until the last follow-up (January 2011). The follow-up period ranged from 4 to 71 months (median 34.6 months). In addition, 45 fresh NSCLC tissues and corresponding noncancerous lung tissues were obtained at our Hospital

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between 2011 and 2012. Of the 45 patients, 26 were stage I-II and 19 were stage III-IV. All protocols were approved by the Hospital Review Board. The informed consent was obtained.

Immunohistochemistry

Formalin-fixed paraffin-embedded specimens were cut into 4 μm-thick sequential sections. Immunostaining was performed using the streptavidin-peroxidase method. Hydrogen peroxide (3%) was applied to block endogenous peroxidase activity, and normal goat serum was used to reduce nonspecific binding. Then sections were incubated with anti-p-mTOR antibody (1:50 dilution) or anti-p-RPS6KB1 antibody (1:100 dilution) (CST) overnight at 4°C. Sections were counterstained with hematoxylin, dehydrated through alcohol, and mounted using a standard procedure. All the samples were evaluated by 2 independent pathologists in the light of the scoring system described previously (Dong et al., 2011). The intensity of p-mTOR or p-RPS6KB1 staining was scored as 0, 1, 2, and 3. Scoring for percentage of positive cells was assigned as 0 (<5%), 1 (5%-25%), 2 (26%-50%), and 3 (≥51%). The scores of each view were multiplied to generate a final score of 0-9, and the final score of one sample was the mean of 10 microscopic fields. Tumors were finally determined as negative (–), score 0; (+), score 1; (++), score 2-4; and (+++), score 6-9. We defined (++) to (+++) as high expression, and (–) to (+) as low expression.

Western blot

Proteins were extracted using cell lysis buffer and concentration was determined by the bicinchoninic acid assay (Promega). Equal volume (60μg) was separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore, USA). After blockade with 5% BSA, membranes were incubated with primary antibodies for p-mTOR (1:1000), p-RPS6KB1 (1:1000) or β-actin (1:2000) (all from CST) overnight at 4°C. After incubation with peroxidase-coupled secondary antibodies (Santa Cruz) at 37°C for 2h, the protein bands were visualized by enhanced chemiluminescence reagent (Thermo Scientific). The optical density of each protein band was measured using the Image Quant software. The β-actin was used to normalize the content of the proteins.

Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde and rinsed in PBS. Cells were blocked with 5% BSA at 37°C for 60 min. The primary rabbit polyclonal antibody for p-mTOR (1:100) or p-RPS6KB1 (1:50) was then added to cells and incubated at 4°C overnight, followed by the incubation with secondary antibodies. The nuclei were counterstained with DAPI at 37°C for 20 min. Fluorescent images were recorded in the same conditions using confocal laser scanning microscope (Olympus).

Statistical analysis

SPSS 16.0 was applied for data processing. χ^2-test and non-parametric test were used to analyze the correlations between p-mTOR or P-RPS6KB1 expression and clinicopathological characteristics. The association between p-mTOR and P-RPS6KB1 expression of the same specimen was analyzed using Spearman rank. Kaplan-Meier curves and log-rank test were used for survival analysis. A multivariate analysis was performed using the Cox regression model to determine the effects of different variables on survival. All data were expressed as mean±SD. $P<0.05$ was considered statistically significant.

Results

Association of p-mTOR and p-RPS6KB1 expression with clinicopathological parameters

Immunohistochemical staining showed that p-mTOR was mainly expressed in the cytoplasm. In NSCLC tissues, the high expression rate of p-mTOR was 48.33% (58 of 120), significantly higher than that in adjacent noncancerous lung tissues ($P<0.01$; Figure 1). The relationship between p-mTOR expression and clinicopathological features is shown in Table 1. The high expression rate of p-mTOR in tumors of stage III-IV (56.3%) was greater than that in stage I (21.1%) or stage II (32.4%, $P<0.01$).The high expression rate of p-mTOR in poor differentiated cancer tissues (66.7%) was greater than that in well (20.1%) and moderate (26.8%) differentiated cancer tissues ($P<0.01$). The high expression rate of p-mTOR was also greater in cases with lymphatic metastasis than in those without lymphatic metastasis (48.6% versus 24%, $P<0.01$). We also examined the expression of P-RPS6KB1 (Figure 1). High expression rate of p-RPS6KB1 was 40.8% (49/120). The high expression rate of p-RPS6KB1 in stages III-IV (39.6%) was greater than that in stages I (31.5%) or II (23.5%, $P<0.01$). High expression rate of p-RPS6KB1 was greater in poor differentiated (64.1%) cancer tissues than that in moderate-well differentiated cancer tissues

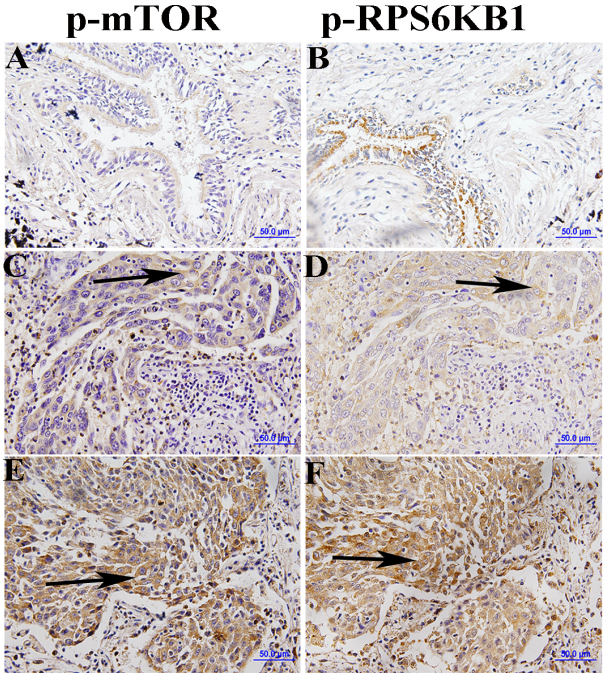

Figure 1. Immunohistochemical Staining of p-mTOR and p-RPS6KB1 in Non-small Cell Lung Cancer (NSCLC) Tissues and Noncancerous Lung Tissues. (A, B) Expression of TOR and P-RPS6KB1 was negative in bronchial epithelium: 0-weak (+); (C, D) ++ in NSCLC; (E, F) +++ in NSCLC

Table 1. Expression Pattern of p-mTOR/p-RPS6KB1 and Clinicopathological Features in Non-small Cell Lung Cancer Patients

Features	Cases (N)	p-mTOR low	p-mTOR high	*P*	p-RPS6KB1 low	p-RPS6KB1 high	*P*
Gender				0.437			0.269
Male	82	48	33		45	36	
Female	38	26	13		26	13	
Age				0.53			0.731
<60years	54	35	19		31	23	
≥60years	66	39	27		40	26	
TNM stage				0.003			0.001
I	38	30	8		26	12	
II	34	23	11		26	8	
III-IV	48	21	27		19	29	
Differentiation				0.001			<0.001
Well	25	20	5		21	4	
Moderate	56	41	15		36	20	
Poor	39	13	26		14	25	
Histological				0.78			0.163
Squamous cell	63	38	25		33	30	
adenocarcinoma	57	36	21		38	19	
Nodal status				0.006			0.097
N0	51	38	12		34	16	
N1 N2 N3	69	36	34		37	33	

Table 2. Multivariate prognostic analysis of non-small cell lung cancer patients

Variate	B	SE	Wald	*p*	HR	95% CI for HR	
						Lower	Upper
Histological type	–0.223	0.183	1.478	0.264	0.8	0.559	1.146
Gender	0.641	0.352	3.309	0.079	1.898	0.952	3.784
History smoking	0.478	0.292	2.671	0.113	1.613	0.909	2.861
Tumor size	0.074	0.258	0.083	0.786	1.077	0.649	1.787
Position	–0.019	0.237	0.007	0.947	0.981	0.617	1.56
Differentiation	–0.047	0.243	0.037	0.843	0.954	0.592	1.538
TNM stage	0.106	0.169	0.392	0.501	1.111	0.798	1.547
Lymph node status	0.567	0.238	5.653	0.016	1.792	1.107	2.813
Distant metastasis	1.332	0.278	22.898	0	3.789	2.196	6.539
p-mTOR expression	1.892	0.507	13.984	0.002	2.642	1.157	4.904

B means partial regression coefficient; SE means partial regression coefficient standard errors. Wald means (B/SE) 2; HR means hazard ratio; 95%CI means 95%; CI means confidence interval

(16% and 35.7%, $P<0.01$). A significant correlation was found between p-mTOR and p-RPS6KB1 expression by Spearman correlation analysis (r=0.58, $P<0.01$).

In order to verify the results of immunohistochemistry, we detected the protein expression of p-mTOR and p-RPS6KB1 by Western blot in 45 fresh NSCLC tissues and paired noncancerous tissues. As a result, protein levels of p-mTOR and p-RPS6KB1 were significantly higher in NSCLC specimens than in noncancerous specimens (Figure 2C).

p-mTOR/p-RPS6KB1 expression was associated with prognosis of NSCLC patients

The overall survival was significantly lower in patients with high expression of p-mTOR than in patients with low expression of p-mTOR ($P<0.01$; Figure 2A). In addition, the overall survival was also significantly lower in patients with high expression of p-RPS6KB1 than in patients with low expression of p-RPS6KB1 ($P<0.01$; Figure 2B).

Cox proportional hazard multivariate analysis

p-mTOR/P-RPS6KB1 expression in NSCLC tissues was analyzed together with its association with prognosis

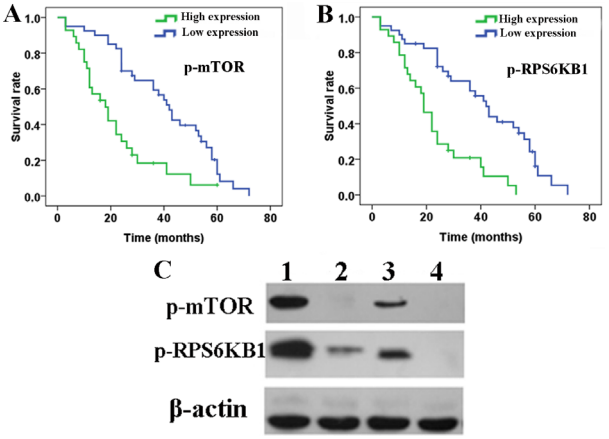

Figure 2. Kaplan–Meier Survival Curves of Non-small Cell Lung Cancer (NSCLC) Patients. (A) Stratified according to p-mTOR expression (log-rank $P<0.01$). (B) Stratified according to p-RPS6KB1 expression (log-rank $P<0.01$). (C) Western blot analysis on the expression of p-mTOR and p-RPS6KB1 in squamous cell carcinoma (Lane 1); normal lung tissue (Lane 2), adenocarcinoma (Lane 3) and normal lung tissue (Lane 4). We detected the protein expression of 45 fresh NSCLC tissues and paired noncancerous tissues. The results were representative of three independent experiments

and clinicopathological features including histological types, sex, smoking history, tumor size, differentiation, metastasis status and TNM stage. Multiple variables were included in Cox proportional hazards analysis. The results showed that lymph node status (HR=1.792, 95%CI 1.107-2.813, $p=0.013$), distant metastasis (HR=3.789, 95%CI 2.196-6.539, $p<0.001$) and p-mTOR expression (HR=2.642, 95%CI 1.157-4.904, $p=0.002$) were independent prognostic indicators for patients with NSCLC (Table 2).

Discussion

In the present study, we determined the expression of p-mTOR and p-RPS6KB1 in human NSCLC cancerous and noncancerous tissues. Our study revealed several novel findings. First, the results showed that there was higher expression of p-mTOR and p-RPS6KB1 in NSCLC than in adjacent noncancerous tissues (negative or weak), indicating that p-mTOR and p-RPS6KB1 can be potential biomarkers to distinguish cancer from noncancerous lesions. Second, high expression level of p-mTOR and p-RPS6KB1 was significantly associated with poor survival of the patients. Moreover, p-mTOR was an independent prognostic factor. All this suggests that p-mTOR and p-RPS6KB1 can be regarded as predictive markers for NSCLC patients and the combination detection of the two markers may be more sensitive.

The mTOR/RPS6KB1 pathway has recently attracted increasing attention in various cancers. Previous evidence has shown that dysregulation of the PI3K/AKT/mTOR pathway offers a favorable oncogenic environment for a variety of human tumors (Huang et al., 2003). The PI3K/AKT signaling pathway activates mTOR, which, in turn, directly phosphorylates ribosome protein RPS6KB1 (also termed S6K1), which is critical in the control of protein translation initiation (Ma et al., 2009). mTOR is

constitutively activated in a variety of human cancers, including ovarian, pancreatic and lung carcinoma (Shaw et al., 2006). Indeed, the mTOR inhibitor Rapamycin and its analogs have been designed to treat human cancers. Data have validated the importance of mTOR inhibition as a profound treatment strategy for several malignancies including renal cell carcinomas, neuroendocrine tumors, pancreatic islet cell tumors, breast cancer, hepatocellular carcinomas, and gastric cancer (Matsubara et al., 2012). Similarly, various studies have shown that increased mTOR activity contributes to lung cancer growth through AKT activation (Tsurutani et al., 2006) and K-ras mutation (Friday et al., 2005).

In this study, we determined whether mTOR and its downstream factor RPS6KB1 were involved in NSCLC. As expected, the expression level of p-mTOR and p-RPS6KB1 indicative of mTOR activation was significantly higher than normal controls by immunohistochemistry and Western blot. p-mTOR expression correlated with p-RPS6KB1. RPS6KB1, key downstream of mTOR activation, has received much more attention in tumor biology. RPS6KB1 is a potential biomarker for aggressive diffuse large B-cell lymphomas (Zhao et al., 2013). RPS6KB1 activation contributes to breast cancer (Baxi et al., 2012). And the RPS6KB1 has been proposed as the treatment target for ovarian cancer (IP et al., 2012).

In conclusion, our data suggest that p-mTOR and p-RPS6KB1 have great potential as prognostic markers in NSCLC patients. Combined detection of p-mTOR and p-RPS6KB1 may be used to evaluate the degree of malignancy in NSCLC. More large-scale prospective studies are warranted to confirm the findings.

References

Baxi SM, Tan W, Murphy ST, Smeal T, Yin MJ (2012). Targeting 3-phosphoinoside -dependent kinase-1 to inhibit insulin-like growth factor-I induced AKT and p70 S6 kinase activation in breast cancer cells. *PLoS One*, **7**, e48402.

Chen J, Fang Y (2002). A novel pathway regulating the mammalian target of rapamycin (mTOR) signaling. *Biochem Pharmacol*, **64**, 1071-7.

Dong QZ, Wang Y, Dong XJ, et al (2011). CIP2A is overexpressed in non-small cell lung cancer and correlates with poor prognosis. *Ann Surg Oncol*, **218**, 857-65.

Ferrara N, Gerber HP, LeCouter J (2003). The biology of VEGF and its receptors. *Nat Med*, **9**, 669-76.

Friday BB, Adjei AA (2005). K-ras as a target for cancer therapy. *Biochim Biophys Acta*, **1756**, 127-44.

Huang S, Houghton PJ (2003). Targeting mTOR signaling for cancer therapy. *Curr Opin Pharmacol*, **3**, 371-7.

Ip CK, Wong AS (2012). Exploiting p70 S6 kinase as a target for ovarian cancer. *Expert Opin Ther Targets*, **16**, 619-30.

Jemal A, Siegel R, Xu J, Ward E (2010). Cancer statistics, 2010. *CA Cancer J Clin*, **60**, 277-300.

Ma XM, Blenis J (2009). Molecular mechanisms of mTOR-mediated translational control. *Nat Rev Mol Cell Biol*, **10**, 307-18.

Malik PS, Sharma MC, Mohanti BK, et al (2013). Clinico-pathological Profile of Lung Cancer at AIIMS: A Changing Paradigm in India. *Asian Pac J Cancer Prev*, **14**, 489-94.

Matsubara H, Sakakibara K, Kunimitsu T, et al (2012). Non-small cell lung carcinoma therapy using mTOR-siRNA. *Int J Clin Exp Pathol*, **5**, 119-25.

Menon S, Manning BD (2008). Common corruption of the mTOR signaling network in human tumors. *Oncogene*, **27**, S43-51.

Minna JD, Roth JA, Gazdar AF (2002). Focus on lung cancer. *Cancer Cell*, 1, 49-52.

Shaw RJ, Cantley LC (2006). Ras, PI(3)K and mTOR signalling controls tumour cell growth. *Nature*, **441**, 424-30.

Thomas A, Rajan A, Lopez-Chavez A, Wang Y, Giaccone G (2013). From targets to targeted therapies and molecular profiling in non-small cell lung carcinoma. *Ann Oncol*, **24**, 577-85.

Tsurutani J, Fukuoka J, Tsurutani H, et al (2006). Evaluation of two phosphorylation sites improves the prognostic significance of Akt activation in non-small-cell lung cancer tumors. *J Clin Oncol*, **24**, 306-14.

Wang D, Chen J, Guo F, et al (2011). Clinical significance of p-mTOR and p-mTOR protein expression in human colorectal carcinomas. *Asian Pac J Cancer Prev,* **12**, 2581 -4.

Zhao XF, Zhao MY, Chai L, et al (2013). Amplified RPS6KB1 and CDC2 genes are potential biomarkers for aggressive HIV+/EBV+ diffuse large B-cell lymphomas. *Int J Clin Exp Pathol*, **6**, 148-54.