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Original Article

Expression of RSK4 in lung adenocarcinoma tissue and its clinicopathological value: a study based on RNA-seq data and immunohistochemistry

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Abstract: The clinical significance of p90-kDa ribosomal S6 kinase (RSK) family members in lung adenocarcinoma (LUAD) is unknown. The aim of this study was to analyze genomic alterations in RSK family membersand investigate the expression and clinicopathological significance of RSK4 in LUAD tissues. Genomic calterations of RSK family, overall survival, andinteraction networks were analyzed in patients with LUAD (n=522), using data from The Cancer Genome Atlas (TCGA) database. The expression of RSK4 protein was investigated in an independent cohort of patients with LUAD (n=127) by immunohistochemistry, and relationships between RSK4 protein levels and clinicopathological parameters of LUAD were analyzed. Patients with LUAD with RSK genomic alterations had significantly better overall survival than patients without alterations (P=0.026). *RSK4* mRNA was overexpressed in LUAD compared with noncancerous lung tissue (P=0.013), and the diagnostic value of *RSK4* expression was moderate, as evaluated by a value of 0.603 for the area under the receiver operator characteristic curve (95% CI, 0.551-0.655; P=0.01). RSK4 protein levels were significantly higher in LUAD than in para-carcinoma tissue (P=0.002); moreover, RSK4 protein expression was closely associated with TNM stage (P<0.001), tumor diameter (P<0.001), lymphatic metastasis (P<0.001), and distant metastasis (P=0.003). In conclusion, alterations to RSK family members may affect tumor progression in LUAD. RSK4 is a potentially useful molecular biomarker for LAUD diagnosis and may predict disease progression in patients with this condition.

Keywords: Lung adenocarcinoma, RSK family, RSK4, TCGA, immunohistochemistry

Introduction

Lung cancer remains the main cause of death from malignant tumors worldwide, with both the highest number of patients surviving with tumors and the highest mortality rates [1]. Although progress has been made in early detection and standard treatments, lung cancer still has high recurrence (14%) and mortality (27%) rates, owing to poor understanding of its pathogenesis. Non-small cell lung cancer (NSCLC) consists of three major histological subtypes: squamous cell carcinoma (LUSC), adenocarcinoma (LUAD), and large cell carcinoma. NSCLC is the most common histologic subtype among lung tumors, accounting for almost 85% of lung carcinoma [2]. Identification of sensitive and specific biomarkers with clinicopathological and prognostic significance is necessary for improved treatment of NSCLC.

Proteins of the 90-kDa ribosomal S6 kinase (RSK) family are serine and threonine protein kinases that function downstream of the Ras/ERK/MAPK pathway. There are four family members: RPS6KA1 (RSK1), RPS6KA3 (RSK2), RPS6KA2 (RSK3), and RPS6KA6 (RSK4). Activated RSKs are associated with multiple pathologies in various types of disease; for example, angiocardiopathy, infectious diseases, and hepatic and pulmonary fibrosis [3]. RSK proteins are remarkable in that they possess two functional

Table 1. RSK4 expression in lung adenocarcinoma and normal tissue

Tissue	N	RSK4-negative N (%)	RSK4-positive N (%)	Z	P
Normal lung tissue	30	26 (86.7)	4 (13.3)		
Adenocarcinoma	127	72 (56.7)	55 (43.3)	-3.039	0.002
Acinar adenocarcinoma	83	49 (59.0)	34 (41.0)	-2.733	0.006
Bronchioalveolar cell carcinoma	18	10 (55.6)	8 (35.4)	-2.385	0.017
Papillary adenocarcinoma	19	8 (42.1)	11 (57.9)	-3.264	0.001
Mucinous carcinoma	7	5 (71.4)	2 (28.6)	-0.971	0.331

kinase domains, i.e., a carboxyl terminal kinase domain and an amino terminal kinase domain [4], which are highly conserved with 73%-80% nucleotide sequence identity between family members [5]. Silencing of *RSK1* increases the likelihood of lung cancer metastasis *in vivo* and *in vitro*, indicating that it may function as a tumor suppressor gene in patients with lung cancer [6], whereas *RSK2* has been reported to be a oncogene and is associated with malignant processes in human cancers, including glioblastoma [7], osteosarcoma [8], and head and neck squamous cancer [9]. Ribosomal S6 kinase *RPS6KA6* (*RSK4*) maps to the Xchromosome (Xq21.1) and differs from other RSK proteins in that it has been reported to inhibit tumor cell proliferation [10] and to function in cellular survival and differentiation via substrate phosphorylation [11].

To date, the exact role of RSK4 in LUAD remains unclear. In this study, we used RNA-seq data from The Cancer Genome Atlas (TCGA) database and performed immunohistochemistry analysis of RSK4 expression in 127 LUAD and 30 normal lung tissue samples to explore the clinical significance of this member of the RSK family. We also investigated correlations between RSK4 expression and clinicopathological parameters in patients with LUAD.

Materials and methods

TCGA data and CBioPortal source analysis of the RSK family in LUAD

Using the CBioPortal website (http://cbioportal.org), part of the TCGA database [12], we analyzed data to determine the percentage of genomic alterations in RSK family members in samples from patients with LUAD. OncoPrint schematics for the survival indices, overall survival (OS), and disease-free survival (DFS) were generated for RSK family alterations in LUAD directly from CBioPortal (TCGA, provisional data). The analysis included data from 522 patients selected via mRNA expression Z-Scores. An interaction network for RSKs was produced using the CBioPortal website. Original LUAD and noncancerous tissue sample data were also downloaded from TCGA database for further analysis. Furthermore, GEPIA was also used to validate the expression data of RSK4 in LUAD.

Sample collection and study design

In this study, LUAD tissue samples were collected from 127 cases (age 19-84 years). Disease subtypes were determined and were summarized in **Table 1**. LUAD samples were randomly obtained from patients at the First Affiliated Hospital of Guangxi Medical University from January 2010 to December 2012, and normal lung tissue samples were also collected from 30 cases (age 19-73 years) without any lung disease from March 2009 to November 2012 in the same hospital. Ethics approval for this project was obtained from the committee of the First Affiliated Hospital of Guangxi Medical University and informed consent was obtained from each patient for tissue collection and use in further research. All tissue samples were subject to diagnostic review by two pathologists for exact confirmation of disease type.

Immunohistochemistry staining

Expression of RSK4 was detected by immunohistochemistry. LUAD tumor tissue was collected and fixed in 10% neutral-buffered formalin for 48 h. One pathologist (Ping Li) screened hematoxylin and eosin (H and E)-stained sections and analyzed paraffin-embedded tissue samples. Tissue microarray was performed as previously described [13]. Mouse monoclonal anti-human RSK4 antibody (RSK4 PL-68; Santa Cruz Biotech Company, CA, USA) was used at a dilution of 1:300, and other reagents used for immunohistochemical staining were supplied by Shanghai Changdao Biotech Company and

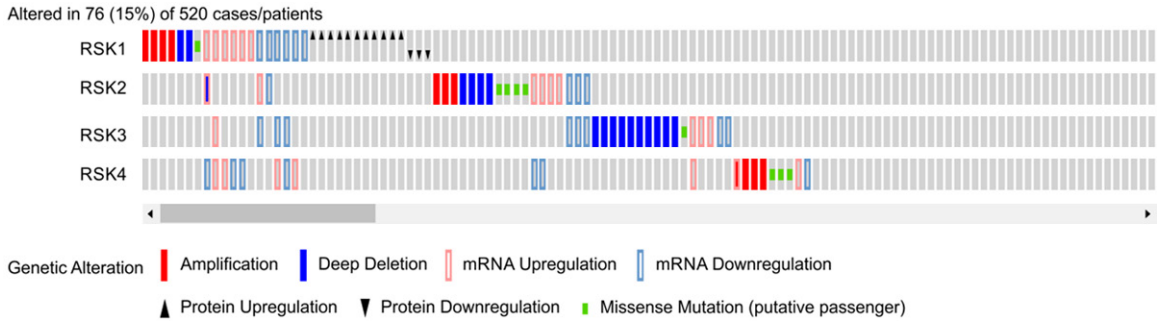

Figure 1. Genomic alterations in RSK family members in 522 lung adenocarcinoma samples (LUAD; TCGA, provisional), selected mRNA expression Z-scores (determined by microarray). Gene amplification, mRNA upregulation/downregulation, protein upregulation/downregulation, and missense mutations of RSK family members (RSK1, RSK2, RSK3 and RSK4) in LUAD are shown. The schematic was generated using OncoPrint via CBioPortal (www.cbioportal.org).

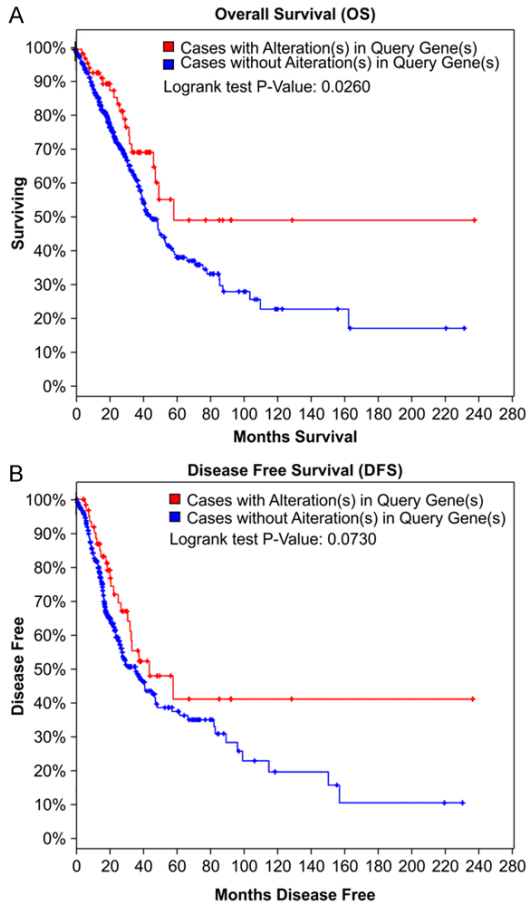

Figure 2. Relationship between genomic alterations of RSK family members and patient survival in 522 lung adenocarcinomas (LUAD, TCGA, provisional), with selected mRNA expression Z-scores (determined by microarray). A. Overall survival (OS): 20 cases were deceased among 75 cases with RSK alterations (median survival time, 58.8 months); 164 cases were deceased among 435 cases without RSK alterations (median survival time, 45.3 months); logrank test, P=0.026. B. Disease-free survival (DFS): 24 cases relapsed among 66 cases with RSK alterations (median time disease-free, 44.02 months); 162 cases relapsed among 368 cases without RSK alterations (median time disease-free, 35.58 months); logrank test, P=0.0729. Survival was analyzed by Kaplan-Meier estimate, using CBioPortal (www.cbioportal.org).

used according to the manufacturer's instructions. All samples were then re-examined and analyzed by two experienced pathologists (Ping Li and Gang Chen), who were blind to the initial diagnosis and outcomes of all patients. The average percentages of positive cells were scored using the following scale: 0, 0%; 1, 1%-25%; 2, 26%-50%; 3, 51%-75%; and 4, 76%-100%. The intensity of staining was classified as follows: 0, negative; 1, weak; 2, moderate; 3 or 4, strong. The expression of RSK4 was measured according to the staining intensity based on the percentage of positively stained cells. Finally, expression scores >2 were considered positive for RSK4 staining. Furthermore, the protein expression data from Proteinatlas was also downloaded to verify the in-house immunohistochemical finding.

Statistical analyses

All statistical data were analyzed using SPSS-20.0 software. Differences in RSK4 protein expression among histological tumor types, pathologic stages, and tumor grades were evaluated using Kruskal-Wallis *H*-tests. A chi-squared test was used to compare RSK4 expression levels in different groups classified according to the following parameters: age, sex, tumor stage (TNM), tumor diameter, lymph node metastasis

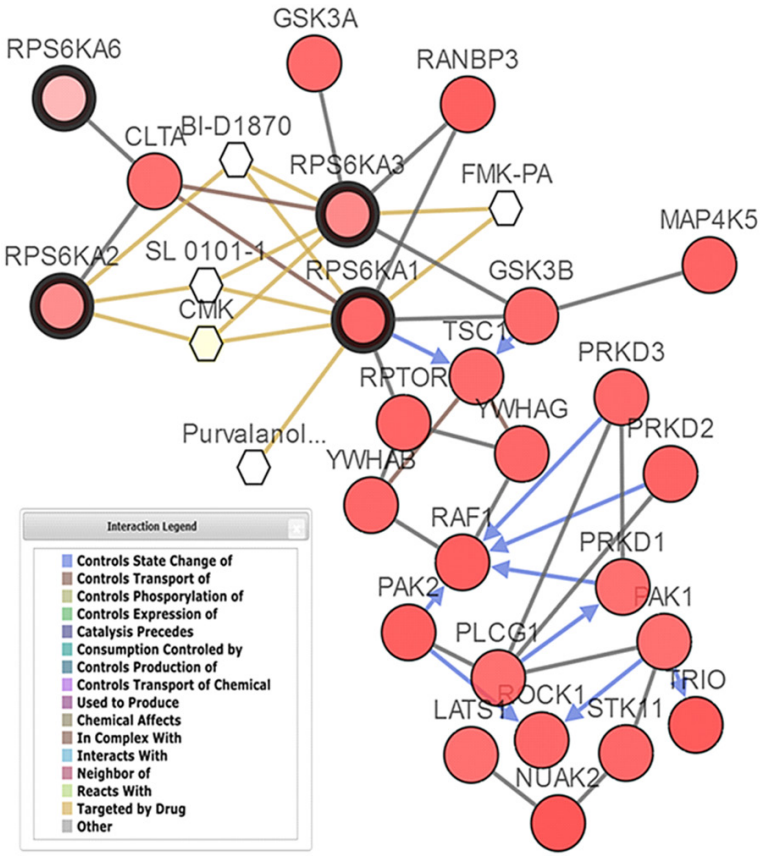

Figure 3. Drugs affecting specific networks related to RSK family members in 522 lung adenocarcinoma (LUAD; TCGA, provisional) samples, with selected mRNA expression Z-scores (determined by microarray). The network in LUAD involving RSK family members (*RPS6KA1* (RSK1), *RPS6KA3* (RSK2), *RPS6KA2* (RSK3), and RPS6KA6 (RSK4)) was generated using CBioPortal (www.cbioportal.org). Circles indicate genes and hexagons represent drugs. *RPS6KA1* (RSK1) is targeted by BI-D1870, FMK-DA, SL0101-1, CMK, and Purvalanol A. *RPS6KA2* (RSK2) is targeted by SL0101-1, BI-D1870, and CMK. *RPS6KA3* (RSK3) is targeted by BI-D1870, FMK-DA, SL0101-1, and CMK, none of which has yet been approved by the FDA.

(LNM), and distant metastasis. Relationships between RSK4 expression levels and clinicopathological features were investigated using Spearman's rank correlation analysis. The value of RSK4 protein expression for lung cancer diagnosis was investigated using a receiver operator characteristic curve (ROC). Results achieving a two-sided significance level of $P< 0.05$ were considered statistically significant.

Results

Bioinformatical analysis of RSK family members using CBioPortal

To explore genomic alterations of all RSK family members and their clinical value in LUAD, we retrieved relevant data from the CBioPortal website. The results demonstrated that genomic alterations in the RSK family (RSK1, RSK2, RSK3, and RSK4) were present in 15% of 520 LUAD cases (**Figure 1**). Patients with LUAD with genomic alterations in RSK family exhibited significantly better OS outcomes than those without such alterations (P=0.026; **Figure 2A**); however, no significant difference was observed in DFS outcomes between the two groups (P=0.0729; **Figure 2B**). To identify the potential molecular mechanism of action of *RSK* family members in LUAD, we generated an interaction network that included all four RSK proteins. The results demonstrated that RSK proteins represent molecular drug targets: RSK1 could be targeted by BI-D1870, FMK-DA, SL0101-1, CMK, and Purvalanol A; RSK3 by BI-D1870, SL0101-1, and CMK; and RSK2 by BI-D1870, FMK-DA, SL0101-1, and CMK. However, none of these drugs were currently approved by the U. S. Food and Drug Administration (FDA) (**Figure 3**).

Data from the TCGA database indicated that RSK4 was overexpressed in LUAD

RSK4 is a vital member of the RSK family, which has been reported to be closely associated with the development of multiple cancers. In our study, RNA-seq data related to RSK4 expression in adenocarcinoma and non-cancerous tissues were extracted from the TCGA database and analyzed. RSK4 was overexpressed in LUAD (n=460) compared with non-cancerous tissues (n=59) ($P<0.05$; **Figure 4A**). In addition, ROC curves were generated to analyze the value of RSK4 expression for distinguishing LUAD from normal lung tissue, and the resulting area under the curve was 0.603 (95% CI, 0.551-0.655; P=0.01). Furthermore, the

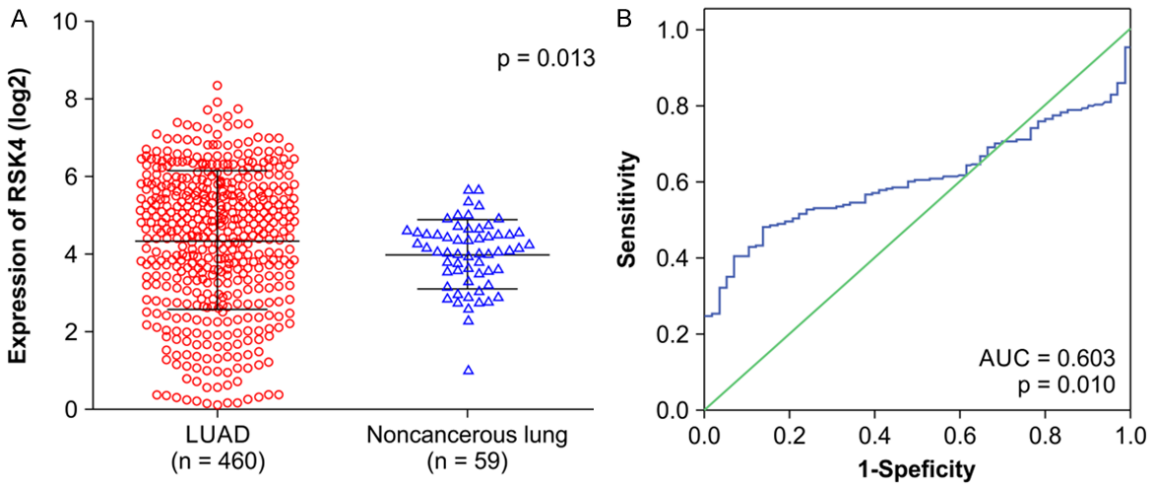

Figure 4. Expression of *RSK4* in lung adenocarcinoma (LUAD), based on RNA-seq data from TCGA database. A. Comparison of *RSK4* expression in LAUD (n=460) and non-cancerous (n=59) lung tissue; *RSK4* expression was elevated in LUAD tissue (P<0.05). B. Receiver operating characteristic (ROC) curves were generated to evaluate the effect of *RSK4* in lung adenocarcinoma. The area under the ROC curve (AUC) for *RSK4* was 0.603 (95% CI, 0.551-0.655; P=0.010).

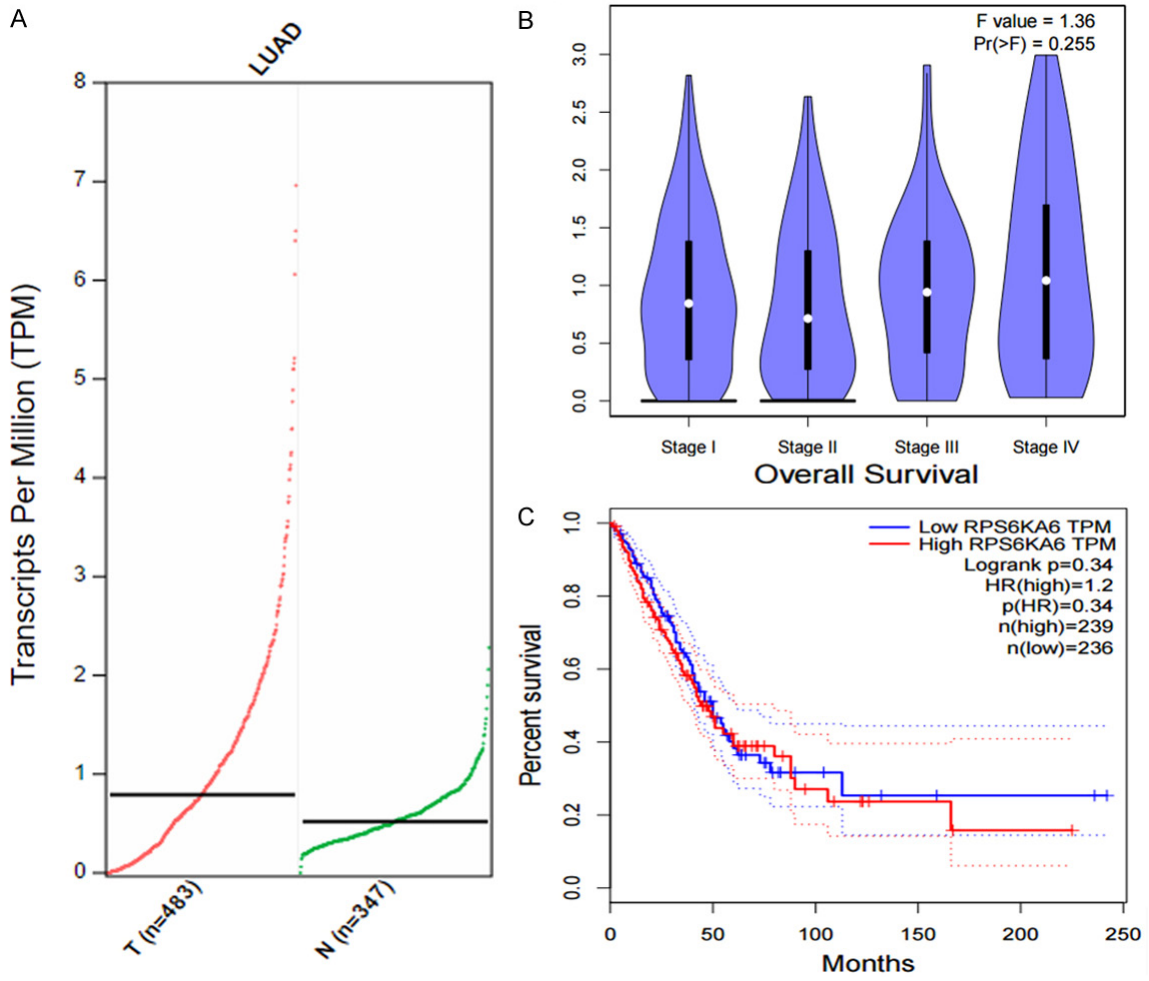

Figure 5. Expression of *RSK4* mRNA in lung adenocarcinoma (LUAD), based on RNA-seq data from TCGA database assessed by TPM from GEPIA. A. Comparison of *RSK4* expression in LUAD (n=483) and non-cancerous (n=347) lung tissue. B. *RSK4* mRNA in different stages of LUAD. C. Prognostic role of *RSK4* mRNA in LUAD.

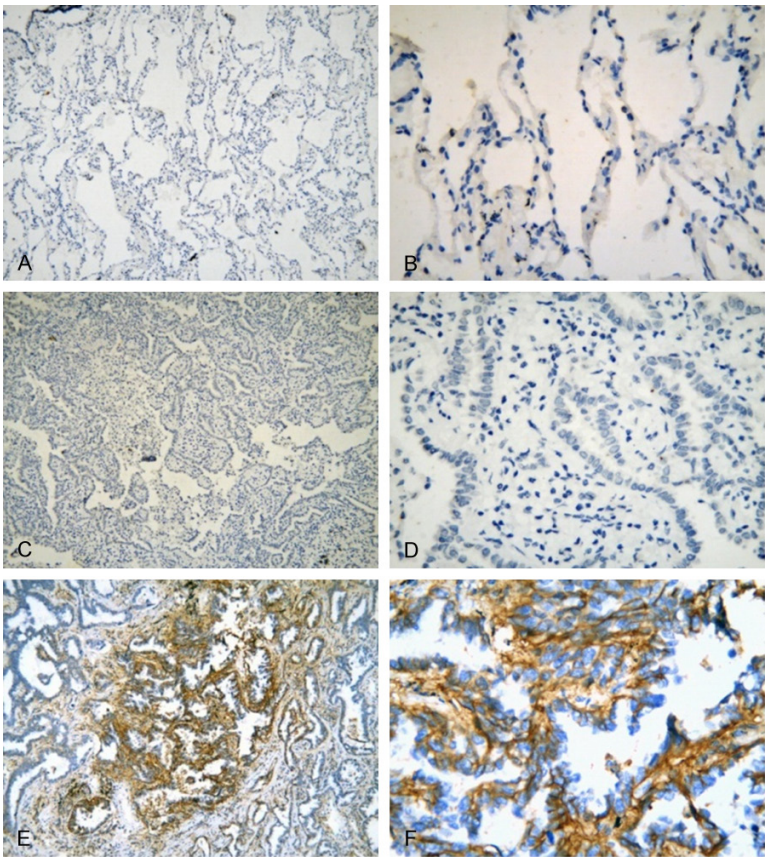

Figure 6. Representative in-house immunohistochemistry images of lung adenocarcinoma samples stained for RSK4 expression. Negative staining for RSK4 in normal lung tissue (A, ×100; B, ×400). Negative staining for RSK4 in adenocarcinoma (C, ×100; D, ×400). Positive staining for RSK4 in lung adenocarcinoma (E, ×100; F, ×400).

RNA-seq data assessed by TPM also indicated that RSK4 was over-expressed in LUAD (**Figure 5**).

RSK4 protein was overexpressed in LUAD tissue

Among 127 LUAD patient samples tested, 55 (43.3%) were RSK4-positive, whereas only four of 30 (13.3%) para-cancerous lung tissue samples were positive (**Figure 6**); the difference between the two groups was statistically significant (P=0.002; **Table 1**). RSK4 expression was then analyzed in LAUD samples separated into four groups based on histological subtype: acinar adenocarcinoma, papillary adenocarcinoma, bronchioloalveolar cell carcinoma, and mucinous carcinoma. Our results indicated notably higher expression of RSK4 in the acinar adenocarcinoma (P=0.006), papillary adenocarcinoma (P=0.001), and bronchioloalveolar cell carcinoma (P=0.017), but not in mucinous carcinoma (P=0.331) (**Table 1**). The over-expression of RSK4 protein level could also be validated by the data from Proteinatlas based on independent cohort (**Figure 7**).

Associations between RSK4 protein expression and clinicopathological parameters of patients with LUAD

Associations between RSK4 protein expression and clinicopathological parameters of patients with LUAD, including TNM stage, LNM, and tumor size, were investigated. Remarkably, higher levels of positive staining for RSK4 expression (80.9%) were detected in tumors of advanced TNM stage (stage III and IV) compared with stage I and II tumors (39.1%; P<0.001). Patients with lymphatic metastasis (91.1%) and large-diameter tumors (≥7 cm; 77.3%) also exhibited significantly higher RSK4 expression than patients without LNM involvement (17.3%) and smaller tumor size (≤7 cm; 36.5%), respectively (P<0.001 for both comparisons). Regarding distant metastasis, the percentage of tumors positive for RSK4 expression was dramatically higher in patients with distant metastasis (9/9, 100%) than in those without (46/117, 39.3%, P<0.001) (**Table 2**). Our results indicate that RSK4 expression was positively correlated with the majority of clinicopathological features of LAUD, including TNM (P<0.001), lymphatic metastasis (P<0.001), tumor diameter (P<0.001), and distant metastasis (P=0.003) (**Table 2**).

Discussion

LUAD is the most common histological type of lung cancer worldwide [14]. Despite some recent advances in treatment for patients with LUAD, the molecular mechanisms underlying LUAD remain unclear. The RSK family of proteins, which includes four isoforms (RSK1-4), is involved in a variety of cellular biological processes, including cell growth and survival [4].

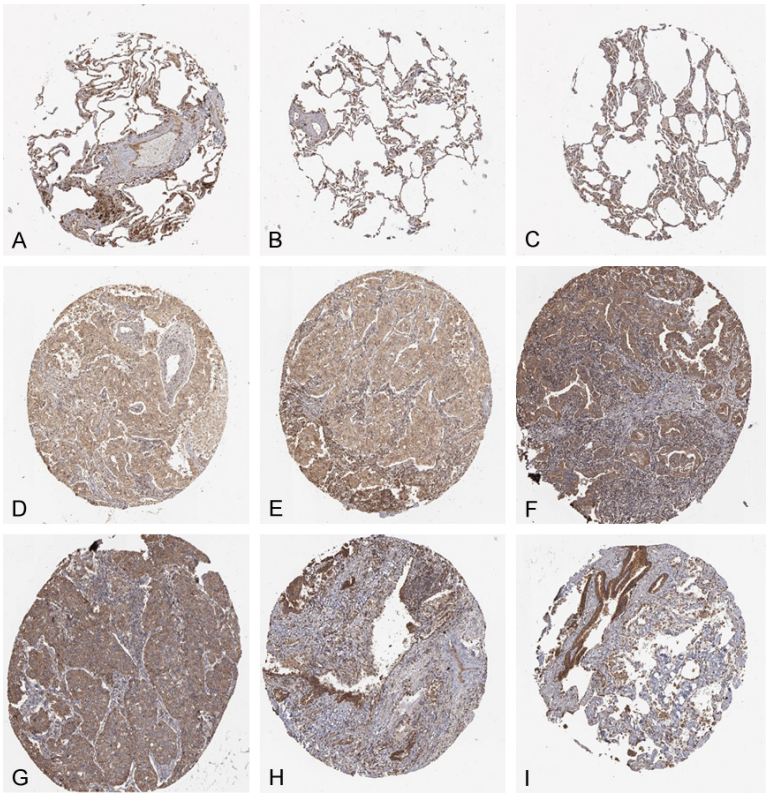

Figure 7. Representative immunohistochemistry images of lung adenocarcinoma samples stained for RSK4 expression from Proteinatlas. Antibody HPA002852 was used. Negative staining for RSK4 in normal lung tissue (A-C ×100), but the staining of macrophages was medium. Medium staining for RSK4 in adenocarcinoma (D-I ×100).

The clinical significance of RSK family members in LUAD has not previously been determined. All genomic alterations involving RSK family members in TCGA data from LUAD samples were identified via CBioPortal. We found genomic alterations in the RSK family in 76 of 520 (15%) patients with LUAD; furthermore, there were significant differences in OS between patients with and without genomic alterations in RSK family. In addition, although no significant difference was found between the two groups in DFS, there was a clear tendency towards a difference between patients with and without genomic alterations (P=0.0729; **Figure 2B**). This inconsistency may be partially attributable to the different sources of samples and the consequent inconsistency of statistical results based on analyses of these public data. A correlation between genomic alterations in RSK family and LUAD has not previously been published; hence, this is the first report that RSK alterations are associated with overall survival in patients with LUAD. Genomic alterations in the RSK family could play important roles in prolonging LUAD patient survival because in patients with renal cell carcinoma (RCC), the expression of *RSK4* has been reported to correlate with poor outcome [15].

The CBioPortal website was also used to generate a RSK family interaction network, providing an intuitive insight into their interactions and incorporating data on gene copy number, mRNA expression, mutations, and alteration frequency. Importantly, the alteration network including RSK family members may provide new directions for future research into LUAD, and indicate specific drugs that could be used to target RSK family members, including BI-D1870, FMK-DA, SL0101-1, CMK, and Purvalanol A. BI-D1870 serves as a potent inhibitor of RSKs both *in vitro* and *in vivo* [16]. In human oral squamous cell carcinoma, BI-D1870 has anticancer activity, affects apoptosis and the cell cycle, and increases the generation of reactive oxygen species [17]; however, the exact molecular mechanism underlying the correlation between changes in RSK proteins and disease prognosis requires confirmation through additional experiments.

In the present study, we evaluated RSK4 expression in LUAD and para-carcinoma samples by TCGA data mining, tissue microarray and immunohistochemical staining. RSK4 is encoded by an X-linked gene and its mutation may cause mental disability in children [15]. The expression of RSK4 is downregulated in colon [18] and breast cancers [19], indicating that it can act as a tumor suppressor gene; however, the function of the RSK4 protein in patients with LUAD has not previously been investigated. Accordingly, the mRNA expression of *RSK4* in LUAD was analyzed in this study for the first time, using data from TCGA database; the expression of *RSK4* mRNA was significantly higher in LUAD than in para-carcinoma samples.

Table 2. Correlation between RSK4 expression and clinicopathological features in all patients with lung adenocarcinoma cancer

Lung adenocarcinoma	N	RSK4-negative N (%)	RSK4-positive N (%)	Z	P
Sex				-0.613	0.540
Male	82	44 (53.7)	38 (46.3)		
Female	45	27 (60)	18 (40)		
Age (years)				-0.043	0.966
<60	52	39 (75)	13 (25)		
≥60	75	32 (42.67)	43 (57.3)		
Pathological grade				4.318	0.115*
I	8	6 (75)	2 (25)		
II	45	29 (64.4)	16 (35.6)		
III	32	14 (43.8)	18 (56.2)		
TNM				-6.721	<0.001
I-II	105	64 (60.9)	41 (39.1)		
III-IV	21	4 (19.1)	17 (80.9)		
LNM				-7.975	<0.001
Yes	45	4 (8.9)	41 (91.1)		
No	81	67 (82.7)	14 (17.3)		
Tumor diameter (cm)				-3.486	<0.001
≤7	104	66 (63.5)	38 (36.5)		
≥7	22	5 (22.7)	17 (77.3)		
Distant metastasis				-3.523	<0.001
No	117	71 (60.7)	46 (39.3)		
Yes	9	0 (0)	9 (100)		

LNM, lymph node metastasis; pathological grading: I vs. II, z=-0.575 (P=0.565); I vs. III, z=-1.561 (P=0.118); II vs. III, z=-1.790 (P=0.073). No significant difference was observed in RSK4 expression relative to other parameters. *Calculated using Kruskal-Wallis H test.

RSK4 expression was found to have moderate value for LUAD diagnosis, with an AUC of 0.603 calculated by ROC curve analysis. Furthermore, we investigated the protein expression of RSK4 in LUAD tissues and para-carcinoma tissues by immunohistochemistry and its correlation with clinicopathological features of LUAD. Similar to the results of mRNA expression analysis, the findings indicated that RSK4 was significantly up-regulated in LUAD compared with para-carcinoma tissues as evidenced by in-house immunohistochemistry and data from Proteinatlas. When the data were analyzed according to LUAD subtypes, RSK4 expression was upregulated in acinar adenocarcinoma, bronchioloalveolar cell carcinoma, and papillary adenocarcinoma, but not in mucinous carcinoma. Hence, results generated using both bioinformatical analyses of data from the TCGA database and immunohistochemistry staining were consistent in demonstrating over-expression of RSK4 in lung adenocarcinoma compared with noncancerous tissues. In line with our results, Fan et al. reported that RSK4 was over-expressed in RCC, and that RSK4 expression was related to high tumor stage, high Fuhrman grade, LNM, and distal metastasis, and could predict poor outcome in patients with RCC. Moreover, overexpression of RSK4 accelerated the course of the cell cycle in RCC and enhanced the invasive and metastatic capability of RCC cells [15]. Our results strongly suggest that RSK4 is a potential new molecular biomarker for the prognosis of LUAD, as established for RCC.

The relationship between RSK4 protein expression and diverse clinicopathological features of LUAD was also evaluated. We discovered that higher expression levels of the RSK4 protein correlated closely with TNM stage, tumor diameter, and both lymphatic and distant metastasis. Moreover, the results indicated that patients with advanced TNM stage and those with lymphatic metastasis exhibited markedly higher expression of RSK4 than patients without these characteristics. Based on these results, we speculate that RSK4 may play an important role in the tumorigenesis of LUAD through stimulation of tumor proliferation, and lymph node and distant metastasis. In support of this hypothesis, knockdown of RSK4 inhibited migration and invasion of the lung adenocarcinoma cell line, A549, consistent with our results in patients with LUAD [6]. Nevertheless, we found no association between RSK4 protein expression and other patient and disease characteristics, including age, sex, histopathological type, and pathological grade. Given the close correlations between RSK4 and TNM stage, tumor size, and lymph node and distant metastasis, expression of RSK4 may exacerbate the progression of LUAD. Nevertheless, additional studies are required to confirm the findings reported here.

Despite the lack of previous investigations of RSK4 in lung adenocarcinoma, relevant molecular functions of RSK4 have been elucidated in other diseases and tumor types. Thakur reported that RSK4 acted as a negative regulator of breast cancer cell invasion through up-regulation of claudin-2 and down-regulation of CXCR4 [20]. RSK4 mediates the invasion and metastasis capacity of RCC cells through regulation of CD44 and MMP-9 expression [15]. In breast [10], endometrial [21], and epithelial ovarian-cancers [22], the mechanism underlying RSK4 epigenetic alteration was investigated, revealing that frequent hypermethylation of the *RSK4* promoter was related to carcinogenesis and cancer progression. In a large-scale RNAi screen in human cells, shRNA stargeting RSK4 suppressed p53-dependent growth arrest in fibroblasts, and RSK4 was found to regulate p53-dependent proliferation arrest through the p21cip1 promoter [23]. In addition, RSK4 was reported to regulate the replicative and stress-induced senescence of endothelial progenitor cells. However, the association between microRNAs or long non-coding RNA (lncRNA) and RSK4 has not been explored. Further *in vitro* and *in vivo* studies are needed to clarify the molecular mechanism underlying the effects of RSK4 in LUAD.

Conclusions

In summary, our observations, along with previous data, indicate for the first time that genomic alterations in RSK family members could play a crucial role in the tumorigenesis and progression of LUAD. *RSK4* expression was significantly elevated in LUAD compared with para-carcinoma lung tissue. Overexpression of RSK4 protein was closely correlated with clinicopathological features indicative of malignant progression in lung adenocarcinoma. Our findings strongly indicate that overexpression of RSK4 is a potential biomarker for diagnosis of LUAD and could be useful for prediction of disease progression. The biological mechanism underlying this phenomenon requires further intensive investigation.

Acknowledgements

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Disclosure of conflict of interest

None.

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