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Original Article

Clinicopathological significance of ribosomal protein S6 kinase A6 in lung squamous cell carcinoma: an immunohistochemical and RNA-seq study

Ye-Ying Fang[1*], Fu-Chao Ma[2*], Xu-Li Gan[2], Wen-Qi Luo[3], Rong-Quan He[2], Hui-Min Xie[2], Shi-Yu Li[2], Gang Chen[3], Dan-Ming Wei[3], Xiao-Hua Hu[2]

Departments of [1]Radiotherapy, [2]Medical Oncology, [3]Pathology, First Affiliated Hospital of Guangxi Medical University, Nanning 530021, Guangxi Zhuang Autonomous Region, P. R. China. [*]Equal contributors.

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Abstract: Ribosomal protein S6 kinase A6 (RPS6KA6) is a downstream factor of the ERK-MAPK pathway and has been extensively studied in various types of cancer. However, the role of RPS6KA6 in lung squamous cell carcinoma (LUSC) remains unclear. This study investigated expression of the RPS6KA6 and its clinicopathological correlation with LUSC and explored genetic alterations in the ribosomal protein S6 kinase (RSK) family in LUSC and their impact on the survival of patients. Expression of the RPS6KA6 protein in 175 LUSC samples and 30 normal lung tissues samples was determined by immunohistochemistry (IHC). RPS6KA6 protein expression in the LUSC tissues was significantly higher compared with that in the normal lung tissues (P=0.017). Overexpression of RPS6KA6 protein correlated with tumor size (r=0.260, P=0.001), lymph node metastasis (r=0.683, P<0.001), and TNM stage (r=0.378, P<0.001). RPS6KA6 RNA-seq data were obtained from the Cancer Genome Atlas (TCGA) and ONCOMINE database. RPS6KA6 mRNA expression in the LUSC tissues was significantly higher than that in paired noncancerous samples (TCGA: P=0.005; ONCOMINE: P=0.018). According to the cBioPortal online software, three detecting methods, including Seqv2, Array and U133, identified that the frequency of the genetic alterations in the RSKs in LUSC were 77%, 44%, and 42%, respectively. However, survival analysis of LUSC patients with or without RSKs genetic alterations reached no statistical significance. This study suggests that RPS6KA6 may be an oncogene in LUSC, and that expression of the RPS6KA6 protein is associated with the progression of LUSC. The RSK genes are frequently altered in LUSC, but the alterations have no significant effect on the survival of patients.

Keywords: Lung squamous cell carcinoma, RPS6KA6, immunohistochemistry, genetic alterations

Introduction

Lung cancer remains the most common cancer and the leading cause of cancer death worldwide [1]. Lung squamous cell carcinoma (LUSC) is one of the histological types of non-small cell lung cancer (NSCLC), accounting for approximately 20-30% of lung cancers [2]. Studies have suggested that LUSC is the most frequent subtype observed in male smokers who are older than sixty years of age [3]. The majority of patients are diagnosed in an advanced stage when chemotherapy is the only treatment option. With resistance to current chemotherapeutics, the use of novel therapeutic targets for the treatment of patients with LUSC continues to be a challenge [4].

The 90-kDa ribosomal protein S6 kinase (RSK) family is a group of serine/threonine kinases consisting of four vertebrate isoforms (RPS6KA1, -3, -2, and -6) and two structural homologs, the mitogen-and stress-activated kinases (MSK1 and 2) [5, 6]. As the active substrate downstream of the mitogen-activated protein kinase (MAPK) cascades, the RSK family participates in a wide range of cellular processes, such as proliferation, differentiation, survival, mobility, protein synthesis, and nuclear signaling [7-9]. In cancer, somatic mutations in the genes that encode the substrates of the Ras-MAPK pathway are observed at an overwhelming frequency, driving great efforts to identify inhibitors that block this pathway for cancer therapy. Currently, several agents targeting the

substrates of the MAPK cascades, such as inhibitors of Ras and MEK, have been developed [10-14]. However, these agents are less than ideal for application in the clinic due to their low selectivity, drug resistance and side effects [15]. Therefore, substrates downstream of the MAPK pathway, such as the RSKs, have generated great interest in cancer research related to aberrant MAPK signaling [16, 17]. Indeed, mounting evidence has suggested that RSKs are extensively involved in tumorigenesis, invasion and metastasis, paving a new way for the treatment of cancer [18-20].

RPS6KA6 was initially discovered in the search for the mental retardation gene at Xq21. In contrast to RPS6KA1, -3, and -2, which are ubiquitous in every human tissue tested, expression of RPS6KA6 is much lower and can be detected only in the brain, cerebellum, heart, renal tissue, and skeletal muscle. In addition, RPS6KA6 can maintain constitutive activity even in serum-starved cells independently of growth factor [21]. The special characteristics of RPS6KA6 make it distinct from the other family members. With increasing studies focused on the relationship between RPS6KA6 and cancers, it became questionable whether RPS6KA6 is an oncogene or a tumor suppressor gene. Cai et al. reported that a decreased expression of RPS6KA6 is observed in colorectal cancer and is associated with the clinical-pathological characteristics and overall survival of patients [22]. In addition, RPS6KA6 exhibits down-regulation in acute myeloid leukemia [23], ovarian tumor [24], and breast cancer [25] and may inhibit proliferation, invasion, and metastasis of cancer cells. In addition, its suppressor role can be explained by two hypotheses that posit that RPS6KA6 may be an inhibitor rather than a mediator of growth factor signal transduction in the ERK/MPAK pathway [26] and that it may be a mediator in p53-induced growth arrest [27, 28]. However, Fan et al. described that over-expression of RPS6KA6 is a risk factor in renal cell carcinoma and positively correlates with an advanced stage and a poor prognosis [29]. In contrast to the finding that silencing of RPS6KA1 enhances the metastatic potential of lung cancer, overexpression of RPS6KA6 was observed in more than 50% of primary malignant lung cancers [30]. Therefore, the definite role of RPS6KA6 in lung cancer remains unknown due to a limited number of studies.

In this study, we detected expression of the RPS6KA6 protein in human lung squamous cell carcinoma (LUSC) samples compared to that in normal lung tissues via immunohistochemistry and investigated its relationship with the clinicopathological characteristics. Furthermore, we took advantage of the data from TCGA and ONCOMINE to further validate the different expression levels of RPS6KA6 mRNA in LUSC and we explored genetic alterations in the RSKs in LUSC and their association with the survival of patients.

Materials and methods

Patient and tissue samples

LUSC samples were collected from 175 patients with LUSC (aged 19 to 84 years, mean 56.35 years) treated in the First Affiliated Hospital of Guangxi Medical University, P. R. China between January 2010 and December 2012. All patients did not receive any radiotherapy or chemotherapy before pneumonectomies. Thirty normal lung tissue samples used as control were excised from autopsies (aged 19 to 73 years; mean 54.03 years) without any lung disease enrolled from March 2009 to November 2012 in the same hospital. All patients were informed that their excised tissues were kept at our hospital and used for scientific study and that their individual privacy would be protected. Written informed consent was also obtained. The study was approved by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University. The clinicopathological features of all the tissue samples, including the pathological grading, tumor diameter, TNM staging, and lymph node metastasis, were reviewed and diagnosed by two independent pathologists using a double-blind method.

Immunostaining evaluation

RPS6KA6 protein expression was tested by immunohistochemical method for the 175 LUSC tissues and 30 normal lung tissues. All tissue samples were fixed in 10% neutral-buffered formalin for 48 h, and then embedded into paraffin for IHC staining with routine procedure. Tissue sections were incubated with mouse monoclonal anti-human RPS6KA6 antibody (RPS6KA6 PL-68; Santa Cruz Biotech Company, CA, USA) which was diluted to 1:300. Other reagents were offered by Shanghai Changdao Biotech Company and used according to the

Table 1. RPS6KA6 protein expression in LUSC and normal tissue

Cancer vs normal tissue	Number	RPS6KA6 negative (n, %)	RPS6KA6 positive (n, %)	z	P
				-2.387	0.017
Normal lung tissue	30	26 (86.7)	4 (13.3)		
Squamous cell carcinoma	175	113 (64.6)	62 (35.4)		

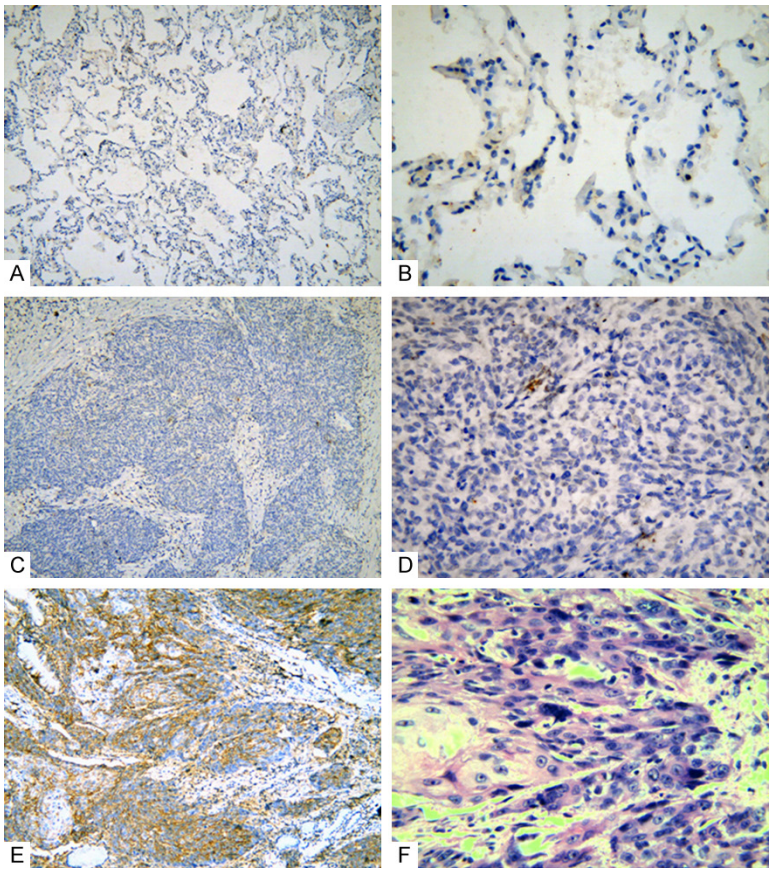

Figure 1. Immunohistochemical photomicrographs of RPS6KA6 in LUSC tissues and normal lung tissues. Negative staining for RPS6KA6 in normal lung tissue (A. ×100; B. ×400). Negative staining for RPS6KA6 in lung squamous cell carcinoma (C. ×100; D. ×400). Positive staining for RPS6KA6 in lung squamous cell carcinoma (E. ×100; F. ×400).

manufacturer's instructions. All samples were assessed independently by two senior pathologists (Ping Li and Gang Chen). Positive cells were counted in 10 randomly chosen high magnification fields (400×). The result was analyzed and graded according to the average percentage and staining intensity of the positive cells. The average percentage of positive cells was recorded as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%). The staining intensity was graded as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The final immunohistochemical outcomes were computed by multiplying the scores of the two aforementioned terms. Scores greater than 2 were regarded as a positive staining.

Public data

Public data of RPS6KA6 mRNA expression were acquired from TCGA and ONCOMINE databases. TCGA database is publicly available and has been used widely by the research community. It collects and characterizes high-quality tumor tissue and matched normal samples from over 11,000 patients. ONCOMINE is a database which currently contains gene expression and sample data from 500 cancer types and a wide range of cancer-related cell lines. The data of ONCOMINE presented to user has been standardized, annotated, and analyzed by Compendia Biosciense. RPS6KA6 mRNA expression of 442 LUSC samples and 51 paired noncancerous samples from TCGA, as well as 27 LUSC samples and 65 paired noncancerous samples from ONCOMINE were analyzed respectively. RPS6KA6 RNA-seq data from TCGA were visualized by GraphPad Prism 5 software packages. While statistical analysis can be performed directly in ONCOMINE. cBioPortal (http://www.cbioportal.org/) is a software that not only provides data from the TCGA but also helps in the analysis and visualization of these data. The frequency of RSK alterations in LUSC was calculated, and a survival analysis was performed using cBioPortal to explore the impact of the RSK alterations on the prognosis of patients with LUSC.

Table 2. Correlation between RPS6KA6 protein expression and the clinicopathological features of LUSC patients

Clinicopathological factor	Number	RPS6KA6 negative (n, %)	RPS6KA6 positive (n, %)	z	p
Gender				-0.247	0.805
Male	148	95 (64.2%)	53 (35.8%)		
Female	27	18 (66.7%)	9 (33.3%)		
Ages (years)				-0.279	0.780
<60	85	54 (63.5%)	31 (36.5%)		
≥60	90	59 (65.6%)	31 (34.4%)		
Pathological grading				0.166*	0.921
I	31	21 (67.7%)	10 (32.3%)		
II	47	30 (63.8%)	17 (36.2%)		
III	97	62 (63.9%)	35 (36.1%)		
TNM					
I-II	148	107 (72.3%)	41 (27.7%)	-4.989	0.000
III-IV	27	6 (22.2%)	21 (77.8%)		
LNM				-9.014	0.000
Yes	57	10 (17.5%)	47 (82.5%)		
No	118	103 (87.3%)	15 (12.7%)		
Tumor diameter				-3.425	0.001
>7	20	6 (30.0%)	14 (70.0%)		
≤7	115	107 (69.0%)	48 (31.0%)		
Distal metastasis				-0.418	0.676
Yes	7	4 (57.1%)	3 (42.9%)		
No	168	109 (64.9%)	59 (35.1%)		

Note: LNM: lymph node metastasis. Pathological grading I vs II: z=-0.353, P=0.724; I vs III: z=-0.387, P=0.699; II vs III: z=-0.010, P=0.992. *: Kruskal-Wallis H test was performed.

Statistical analysis

The SPSS 22 was used for the statistical analyses. Chi-squared test was applied for comparisons between two groups. Kruskal-Wallis H test was adopted for comparisons among three groups. Spearman's rank correlation test was conducted to estimate the correlation between RPS6KA6 expression and the clinical pathological parameters (TNM, distal metastasis, gender, age, tumor size, LNM) in LUSC. For all statistical analyses, P<0.05 represents statistical significance.

Results

RPS6KA6 expression in lung squamous cell carcinoma

Immunohistochemistry analysis revealed that RPS6KA6-positive signaling exist in the cytoplasm and nucleus of the tumor cells. Expression of RPS6KA6 protein in the LUSC patients was significantly higher (35.4%, 62/175) than that in the healthy controls (13.3%, 4/30, P=0.017, **Table 1**; **Figure 1**). Furthermore, the group with larger tumor sizes (>7 cm) showed a more conspicuous expression of RPS6KA6 protein (70.0%, 14/20) compared with the group with smaller tumor sizes (≤7 cm) (31.0%, 48/115, P=0.001). Similarly, a higher positive rate was observed in the group with lymph node metastasis (82.5%, 47/57) compared to that in the group without lymph node metastasis (12.7%, 15/118, P<0.001). Moreover, the expression of RPS6KA6 protein was up-regulated in advanced TNM stages (77.8%, 21/27), which contrasted with the expression in early TNM stage (27.7%, 41/148, P<0.001). However, no significant difference in RPS6KA6 protein expression was observed in the remaining groups. All analyses of the expression of RPS6KA6 protein and the clinical features of LUSC are shown in **Table 2**.

Furthermore, we assessed the associations between RPS6KA6 protein expression and the clinicopathological parameters utilizing Spearman's rank correlation analysis. The results indicate that RPS6KA6 protein expression is correlated with the TNM stage, (r=0.378, P<0.001), tumor size (r=0.260, P=0.001), and lymph node metastasis (r=0.683, P<0.001). The results of the Spearman analysis are presented in **Table 3**.

As to the expression of RPS6KA6 mRNA, it was markedly higher in LUSC samples than in paired noncancerous samples based on TCGA data (P=0.005, **Figure 2**). Compared with noncancerous samples, LUSC samples showed a 1.145 fold change in RPS6KA6 mRNA expres-

Table 3. The correlation between RPS6KA6 protein expression and other clinical pathological parameters in LUSC

RPS6KA6 expression		LUSC
TNM	R value	0.378
	P value	0.000
Distal metastasis	R value	0.032
	P value	0.677
Gender	R value	-0.019
	P value	0.806
Age	R value	-0.021
	P value	0.781
Tumor size	R value	0.260
	P value	0.001
LNM	R value	0.683
	P value	0.000

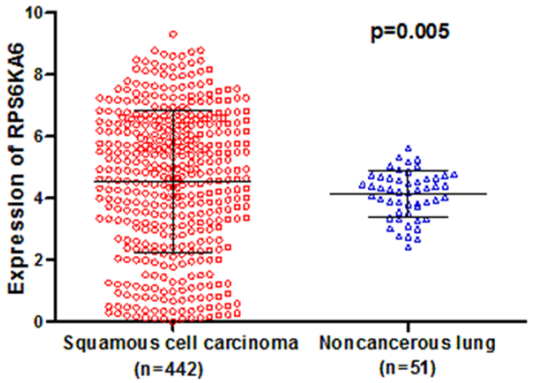

Figure 2. Expression of RPS6KA6 in the cohort form TCGA. Scatter plots shows that RPS6KA6 mRNA is up-regulated in lung squamous cell carcinoma samples (n=442) relative to normal lung samples (n=51).

sion according to the ONCOMINE data (P= 0.018, **Figure 3**).

Genetic alterations in the RSK family in lung squamous cell carcinoma.

In total, we obtained a cohort with 504 cases of LUSC from the cBioPortal database. Three methods, seqv2, array and U133, were used to detect the mRNAs of RSKs and recognize the genetic alterations in the mRNAs. Intriguingly, the rate of RSK genetic alterations in LUSC reached 77% (RPS6KA1: 54%, RPS6KA3: 25%, RPS6KA2: 22%, and RPS6KA6: 16%) by seqv2. In addition, the alteration rate was 44% when an array was adopted (RPS6KA1: 31%, RPS6KA3: 14%, RPS6KA2: 11%, and RPS6KA6: 11%). Furthermore, 42% of the RSK alteration rate was discovered in LUSC using U133 (RPS6KA1: 29%, RPS6KA3: 10%, RPS6KA2: 11%, and RPS6KA6: 8%) (**Figure 4**).

Furthermore, a survival analysis was performed to investigate the relationship between the RSK alterations and the survival of LUSC patients, including the overall survival time and disease free survival time. However, none of the three groups revealed significant differences in the survival of patients with RSK alterations compared to that of patients without RSK alterations (all P>0.05, data were not showed).

Discussion

Chemotherapy is an important treatment option, especially during the advanced stages of cancer when surgery fails to provide a curative effect or a residual focus remains after surgery. In lung cancer, the application of targeted therapeutic agents has been one of the routine treatments for lung adenocarcinoma. However, little benefit from targeted therapies has been gained by patients with LUSC. In fact, the identification of effective targets as chemotherapy agents for LUSC remains elusive.

RPS6KA6 has been extensively studied in various cancers, but its exact role in cancers remains conflicting. A great majority of studies support that RPS6KA6 may play a tumor suppressor role. In addition, multiple analyses of RPS6KA6 expression values detected in clinical samples or cell lines and findings regarding the anti-cancer mechanisms of RPS6KA6 appear to lay the foundation for that perspective. Berns et al. reported that RPS6KA6 may act as a mediator of p53 signaling [28]. Since then, a vast majority of studies attribute the decreased expression of RPS6KA6 that is observed in cancer tissues to that mechanism. In contrast, a study by Bettina A. Dummler later demonstrated that no change in RPS6KA6 was observed either when UV irradiation was given to induce p53 activation or the p53 plasmid infected the cell lines [21]. Moreover, a decrease in the expression of p53 is detected with the overexpression of RPS6KA6 in renal cell carcinoma (RCC), as shown in a study by Fan et al. [29]. Therefore, it appears to be premature to establish the exact mechanism of RPS6KA6 functioning in p53 signaling.

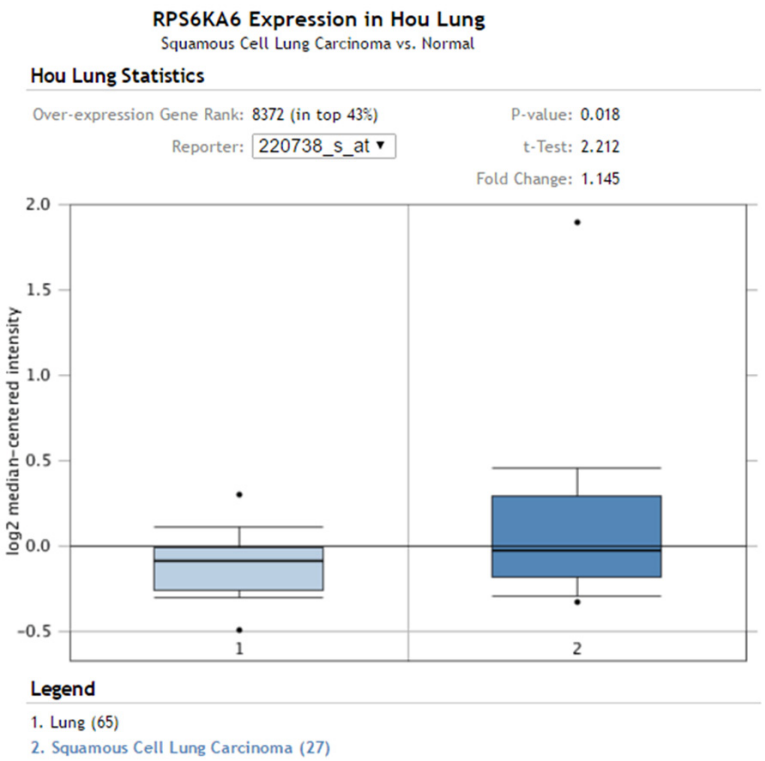

Figure 3. Expression of RPS6KA6 in the cohort form ONCOMINE. Box plots shows that RPS6KA6 mRNA is significantly overexpressed in lung squamous cell carcinoma samples (n=27) compared with normal lung samples (n=65).

Due to the significant controversy and clinical concern regarding this issue, we investigated expression of the RPS6KA6 protein in LUSC tissues compared with that in normal lung tissues by immunohistochemistry. The results show that the positive expression rate of the RPS6KA6 protein in LUSC is significantly higher than that in normal lung tissues. Subsequent analyses with the data of RPS6KA6 mRNA expression from the TCGA and ONCOMINE also show the same trend. These findings suggest that RPS6KA6 might be oncogenic in LUSC, which is supported to some extent by previous observations that RSK4 is overexpressed in more than 50% of malignant lung cancers. In addition, we investigated the correlation between RPS6KA6 protein expression and the clinicopathological parameters. A higher positive expression rate of RPS6KA6 was found in the samples with larger primary tumor sizes (>7 cm), lymph node metastasis, and advanced stages (stage III-IV) compared to that in the corresponding samples of LUSC. The Spearman correlation also showed a positive association between the RPS6KA6 protein level and the tumor size, lymph node metastasis, and TNM stage. Thus, RPS6KA6 may be a promoter of LUSC by facilitating cell proliferation, invasion, and metastasis. However, no significant correlation between RPS6KA6 expression and distal metastasis in LUSC was observed, which was possibly a result of the small number of patients with distal metastasis (n=7).

Recent studies suggest that the frequency of fibroblast growth factor receptor 1 (FGFR1) amplification in LUSC (at least 12%) is significantly higher than that in lung adenocarcinoma (3%) [31, 32]. In fact, mutations in all four FGFR kinases (FGFR1-4) have been described in LUSC [33]. In addition, the FGFR pathway proves to be a hallmark of LUSC. Interestingly, RPS6KA6 participates in the ERK-MAPK pathway, which is the main downstream signaling pathway of FGFR pathway. The mouse RPS6KA6 has been regarded to have the ability to inhibit the FGFR-RAS-ERK pathway, thus inhibiting tumorigenesis. However, L Fan et al. hypothesize that RPS6KA6 may behave as a mediator in the ERK pathway by regulating two cellular adhesion molecules, CD44 and MMP-9, in RCC. The evidence that the precise function of RPS6KA6 may depend on many factors, such as the cell type, allows the role of RPS6KA6 to be diverse in cancer [34]. Therefore, it is tempting to speculate that RPS6KA6 is a tumor promoter mediating the FGFR-ERK-MAPK pathway in human LUSC.

Cancer is basically a genetic disease. The development of cancer is a multistep process, requiring alterations in several cancer-related genes. Since RSKs display the highest protein sequence identity (73-80%) and may share the some of the same functions, we investigated the RSK gene alterations and their impact on LUSC. Information regarding RSK alterations in LUSC was downloaded from cBioPortal, which is a user-friendly interface for accessing and analyzing the TCGA genomics data.

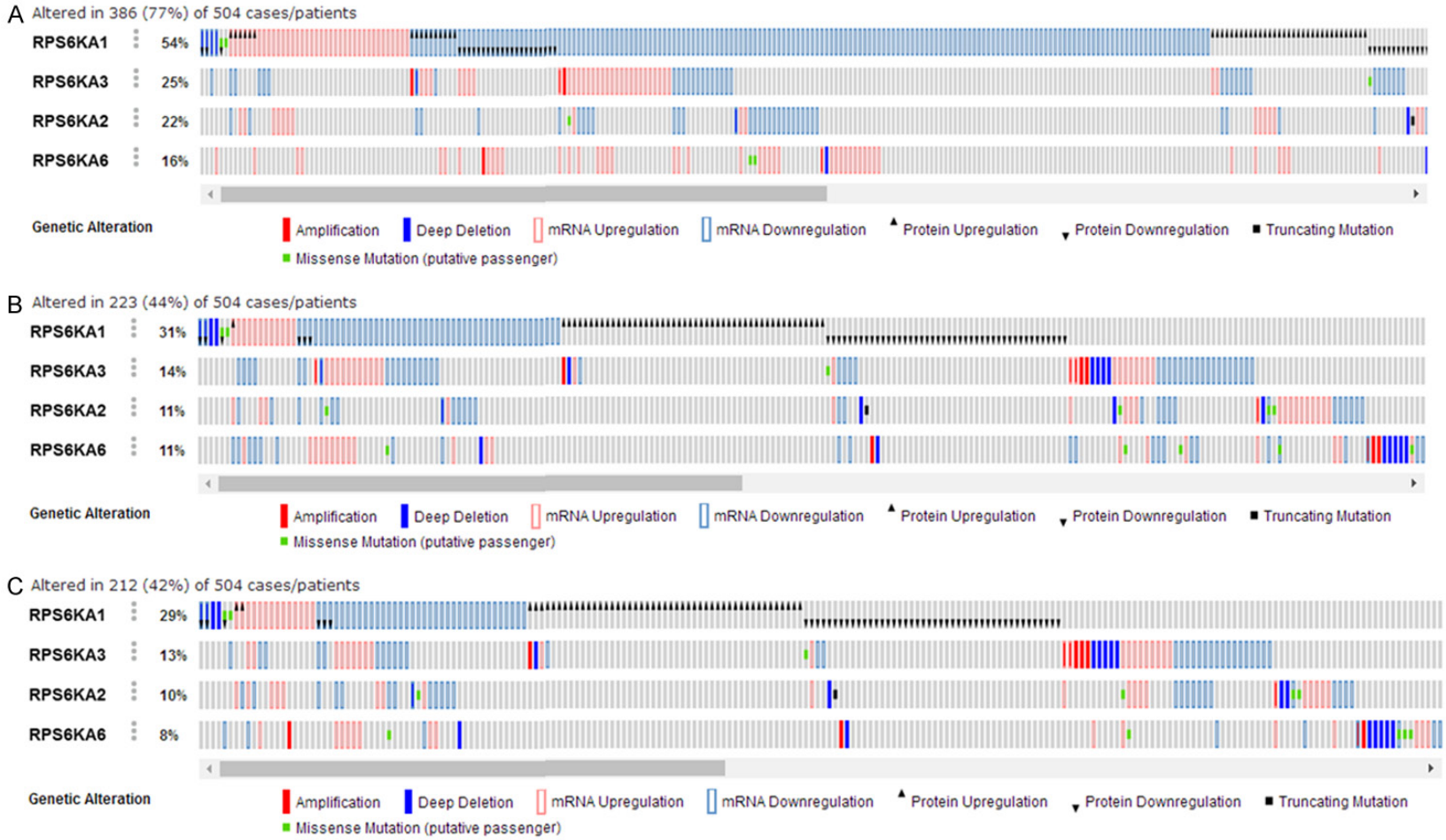

Figure 4. Genetic alterations of RSKs family in 504 lung squamous cell carcinoma cases. RSKs mRNA was detected by RNA Seqv2 (A), Array (B) and U133 (C), respectively.

Surprisingly, of 504 patients with LUSC, 77% were shown to harbor RSK gene alterations via RNA Seq V2 RSEM, 44% via microarray and 42% via only a U133 microarray. Among the alterations in four genes, those in RPS6KA1 account for the majority, followed by those in RPS6KA3, RPS6KA2 and RPS6KA6, successively. However, no significant difference in survival time was observed between the patients with gene alterations in the RSKs and those without alterations in the RSKs.

Metastasis is the primary cause of death in patients with lung cancer; however, the mechanism underlying the tumor dissemination remains poorly understood. In light of previous studies, RSKs have the capacity to regulate the metastatic process, but the regulations may vary according to the type of cancers and the RSK isoforms [35]. The only study that focused on RSKs and lung cancer revealed that silencing RPS6KA1 promotes, while down-regulating RPS6KA3 and RPS6KA6 inhibits, the metastatic potential of lung cancer [30]. Therefore, the overall effect of the ribosomal S6 kinases in lung cancer is complicated, as different isoforms may manifest opposing functions.

Conclusion

We detected the expression level of RPS6KA6 protein using immunohistochemistry and found that the RPS6KA6 expression in LUSC was markedly higher than that in normal lung tissue. Overexpression of the RPS6KA6 protein was positively correlated with primary tumor size, lymph node metastasis, and advanced stages. Additional analyses of the data downloaded from the TCGA and ONCOMINE further validated the higher expression of RPS6KA6 in LUSC than that in normal samples. These findings suggest that RPS6KA6 may play an oncogenic role in LUSC and may be a promising biomarker for the diagnosis and prognosis of LUSC in the clinic. RSKs exhibit frequent gene alterations in patients with LUSC. However, the overall effect of these gene alterations does not lead to a difference in the survival of patients with LUSC, possibly due to the opposing effect of RSKs on LUSC. The underlying mechanisms by which RPS6KA6 promotes LUSC remain unclear with an important clue in that RPS6KA6 may be a mediator in the ERK-MAPK pathway in LUSC. In addition, selective inhibitors of RSK isoforms should be developed to clarify the specific function of each isoform.

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Disclosure of conflict of interest

None.

Address correspondence to: Dan-Ming Wei, Department of Pathology, First Affiliated Hospital of Guangxi Medical University, 6 Shuangyong Road, Nanning 530021, Guangxi Zhuang Autonomous Region, P. R. China. E-mail: danmingwei08@163.com; Xiao-Hua Hu, Department of Medical Oncology, First Affiliated Hospital of Guangxi Medical University, 6 Shuangyong Road, Nanning 530021, Guangxi Zhuang Autonomous Region, P. R. China. E-mail: gxmuhxh@163.com

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