

Skp2 expression has different clinicopathological and prognostic implications in lung adenocarcinoma and squamous cell carcinoma

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Abstract. High expression of S-phase kinase associated protein 2 (Skp2) is associated with numerous clinicopathological parameters, including histology, lymph node metastasis, smoking status, differentiation and Tumor-Node-Metastasis stage in non-small cell lung cancer (NSCLC). Skp2 protein is overexpressed in lung squamous cell carcinoma (LUSC), compared with lung adenocarcinoma (LUAD), whilst the clinicopathological and prognostic implications in LUAD or LUSC remain unclear. A larger study is required to assess the differences in Skp2 expression between these NSCLC types. In the present study, the clinicopathological features and immunohistochemical expression of the Skp2 protein were studied in 500 patients with NSCLC (351 with LUAD and 149 with LUSC). Survival analyses were performed using Kaplan-Meier method and Cox regression model. Skp2 associated genes were identified based on the data from The Cancer Genome Atlas database. Skp2 was overexpressed in patients with LUSC, compared with LUAD ($P<0.001$). In histology subgroup analysis, differences in Skp2 protein expression were observed in patients with LUAD, based on sex, differentiation, smoking history, stage, lymph node metastasis and tumor diameter ($P<0.05$), but not in patients with LUSC except for smoking status. High Skp2 protein expression in patients with LUAD was associated with reduced overall survival (OS; $P<0.001$), but not in patients with LUSC ($P=0.686$). The multivariate analysis demonstrated that Skp2 expression is an independent unfavorable prognostic factor for OS in patients with LUAD ($RR=1.845$, $P<0.05$). Bioinformatics analyses revealed that minichromosome maintenance complex component 2, cell division cycle 45, replication factor C subunit 4,

which are differently expressed in LUAD and LUSC, are associated with Skp2 expression and participate in DNA replication and G₁/S transition. Skp2 protein expression differs in LUAD and LUSC. The clinicopathological and prognostic implications based on Skp2 expression in LUAD and LUSC should be considered different. LUSC with high Skp2 expression may have robust proliferation ability.

Introduction

The F-box protein S-phase kinase associated protein 2 (Skp2), a component of the Skp1-Cullin 1-F-box E3 ubiquitin-ligase complex, promotes the degradation of the cyclin-dependent kinase inhibitor p27, a key regulator of G₁ progression. An inverse correlation between Skp2 and p27 protein level has been reported in tumors (1,2). Under physiological conditions, Skp2 controls the initiation of mitosis in that its expression peaks at the S and G₂ phases, but not at G₀ and G₁ phases (3,4). Skp2 is key in the p27 ubiquitin degradation pathway and may inhibit the proliferation of a variety of cell types through the ubiquitin-proteasome pathway (4-6).

Skp2 overexpression has been observed in various human cancers, including lymphomas (7), prostate cancer (8), melanoma (9), pancreatic cancer (10), gastric cancer (11), breast cancer (12), colorectal cancer (13) and lung cancer (14,15).

The involvement of Skp2 overexpression in metastasis and aggressiveness has been reported in many tumors including melanoma (9), pancreatic cancer (10), breast (12), colorectal cancer (13) and lung cancer (1). Skp2 is also considered to be associated with the occurrence, development and prognosis of malignant tumors such as breast cancer (12), melanoma (9), prostate cancer (8), colorectal cancer (14), and lung cancer (1).

Expression of Skp2 is significantly upregulated in primary non-small cell lung cancer (NSCLC), compared with non-tumorous lung tissues (16). In patients with NSCLC, overexpression of the Skp2 protein is associated with numerous clinicopathological parameters, including histology, lymph node metastasis, smoking status, differentiation and tumor stage (1,16). In addition, the positive expression of Skp2 is correlated with a poor prognosis in patients with NSCLC (1).

Concerning the two major pathological types of NSCLC, Skp2 is notably overexpressed in lung squamous cell carcinoma

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(LUSC), compared with lung adenocarcinoma (LUAD) (1). Due to the limitation of sample amounts, it remains unclear whether Skp2 expression is associated with those clinicopathological features in LUAD and LUSC, respectively; therefore, a study with a greater number of patients and specimens is required to compare the clinicopathological and prognostic implications with the Skp2 expression in these NSCLC types.

In the present study, the correlation between clinicopathological features and Skp2 protein expression was investigated in 351 LUAD and 149 LUSC samples. Additionally, the prognostic value of Skp2 protein expression was also investigated. Bioinformatics analyses were performed to investigate Skp2 expression-associated mRNAs and lung cancer mutations.

Materials and methods

Cases and specimens. Patients were excluded due to chemotherapy or radiotherapy prior to surgery. A total of 351 patients with LUAD and 149 patients with LUSC who were diagnosed and underwent surgery at Peking University People's Hospital (Beijing, China) between January 2004 and December 2012 were enrolled in the present study. The patients included 305 males and 195 females, with a median age of 64 years (range, 24-86). All procedures performed in the current study involving human participants were in accordance with the ethical standards of the Ethics Committee of Peking University People's Hospital. Informed consent was obtained from all individual participants included in the study. All patients were followed up following surgery at 3-6 month intervals, and the total follow-up periods ranged from 4.8 to 7.6 years, with a median of 5.9 years. Complete clinical information from the clinical and pathological records, including sex, age, tumor size, lymphatic invasion, differentiation grade and pathological histology were collected and summarized in Table I.

Normal tissue was collected from at >2 cm away from the primary cancer site and histopathologically identified as normal by a pathologist at the Peking University People's Hospital. Fresh tissue samples from each patient were formalin-fixed (10%) at room temperature overnight, paraffin-embedded and constructed into tissue microarrays (TMA).

TMA and immunohistochemistry (IHC). TMA were prepared using carefully identified representative core-tissue specimens (2 mm in diameter) as previously described (17). Sections 4 μ m thick from the recipient blocks were cut. Antigen retrieval was performed in a pressure cooker, followed by treatment with 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity. IHC analysis was performed using an antibody against Skp2 at room temperature for 2 h (1:200 dilution; cat. no. 2652; Cell Signaling Technology, Beverly, MA, USA). The sections were rinsed and incubated for 30 min with biotinylated second antibody in room temperature. Expression of Skp2 was detected using a diaminobenzidine reaction for 30 min and hematoxylin counterstaining for 30 sec at room temperature. There were two independent pathologists, who were blind to the patients' clinical data, who scored the staining intensity level as follows: Level-0, negative; level-1, weak positive; level-2, moderate positive; level-3, strong positive, and the percentage of positive cells. Skp2 protein expression assessment was based on the method of a semi-quantitatively scoring

system (18). The final score was obtained from multiplying the staining intensity level and the percentage of positively stained cells (A for level-1, B for level-2, C for level-3). The calculation formula was as follows: Score = 1xA% + 2xB% + 3xC%. The semi-quantitative method provided further information regarding the expression of Skp2 and reduced bias, whilst analyzing the correlation with clinicopathological characters.

Skp2 co-expression analysis of LUAD and LUSC differential mRNAs. The Cancer Genome Atlas (TCGA) mRNA-expression data for LUSC and LUAD were obtained from Firehose (<http://gdac.broadinstitute.org/>) using the Bioconductor package RTCGAToolbox (version 2.4.0; <http://bioconductor.org/packages/RTCGAToolbox/>). For LUAD, a provisional dataset of 162 total tumors was used, and for LUSC, 240 samples were used.

Differential mRNAs were screened using the Bioconductor package edgeR (version 3.16.5; <http://bioconductor.org/packages/edgeR/>) (19). The FDR threshold was set to 0.05, in order to identify significantly deviating genes between LUAD and LUSC. The LogFC threshold was set to 1.5 to select mRNAs for the following co-expression analysis. The R statistical language (version 3.4.1; <https://www.r-project.org/>) was used to conduct these analyses.

The spearman rank correlation method was used to assess the co-expression of the differential mRNAs. The top 50 Skp2-associated mRNAs were cluster analyzed and plotted by R package heatmap (version 1.0.8; <https://CRAN.R-project.org/package=heatmap>). The highest associated mRNAs were processed in The Database for Annotation, Visualization and Integrated Discovery functional annotation bioinformatics analysis (20,21).

TCGA gene expression and mutation data for LUAD and LUSC were obtained from the cBioPortal for Cancer Genomics (www.cbioportal.org) (22,23). For LUAD, the provisional dataset of 522 total tumors was used. Mutation data were obtained for tumor protein P53 (TP53), KRAS, Kelch-like ECH-associated protein 1 (KEAP1), serine/threonine kinase 11, epidermal growth factor receptor, neurofibromin 1 (NF1), SET domain containing 2, RNA binding motif protein 10, MGA, MET, AT-rich interaction domain 1A, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (PIK3CA), SWI/SNF-associated matrix-associated actin dependent regulator of chromatin subfamily A member 4, RB transcriptional corepressor 1 (RB1), cyclin dependent kinase inhibitor 2A (CDKN2A), U2 small nuclear RNA auxiliary factor 1, Ras-like without CAAX 1, Erb-B2 receptor tyrosine kinase 2 and ALK. For LUSC, data were obtained from the provisional dataset of 504 samples. Mutation data were obtained for TP53, CDKN2A, phosphatase and tensin homolog (PTEN), PIK3CA, KEAP1, major histocompatibility complex class I A, nuclear factor erythroid 2 like 2 (NFE2L2), NOTCH1 and RB1.

Statistical analysis. Median score and 5-95 percentile were plotted. Skp2 expression among different groups according to pathological grading, lymph node metastasis, smoking status and differentiation grade were evaluated by Kruskal-Wallis H-test which is a non-parametric method for testing whether samples originate from the same distribution. Wilcoxon matched-pairs signed rank test were used to compare Skp2

Table I. Correlation between Skp2 protein expression and clinicopathological parameters in lung cancers.

Histology Clinicopathological parameters	LUAD						LUSC				
	Category	Cases	Skp2 expression		Statistical value	P-value	Cases	Skp2 expression		Statistical value	P-value
			Low	High				Low	High		
Total		351	231	120			149	54	95		
Sex	Male	172	102	70	6.325 ^b	0.012 ^a	133	48	85	0.012 ^b	0.912
	Female	179	129	50			16	6	10		
Age	<65	186	129	57	2.207 ^b	0.137	81	34	47	2.525 ^b	0.112
	≥65	165	102	63			68	20	48		
Differentiation	Well	107	91	16	36.308 ^c	<0.001 ^a	11	7	4	2.898 ^c	0.235
	Moderate	122	78	44			68	25	43		
	Poor	122	62	60			70	22	48		
Smoking status (cigarettes/year)	0	232	163	69	16.198 ^c	<0.001 ^a	31	14	17	6.555 ^c	0.038 ^a
	0<n≤400	62	41	21			30	7	23		
	>400	57	27	30			88	33	55		
T	1	157	110	47	4.410 ^c	0.221	30	9	21	0.653 ^c	0.884
	2	170	110	60			86	35	51		
	3	13	6	7			26	8	18		
	4	11	5	6			7	2	5		
N	0	233	174	59	24.081 ^c	<0.001 ^a	72	23	49	2.564 ^c	0.464
	1	35	17	18			33	13	20		
	2	79	37	42			43	17	26		
	x	4	3	1			1	1	0		
M	0	334	219	115	0.181 ^b	0.670	146	52	94	1.227 ^b	0.268
	1	17	12	5			3	2	1		
TNM stage	I	215	165	50	33.202 ^c	<0.001 ^a	54	19	35	1.127 ^c	0.771
	II	44	19	25			41	14	27		
	III	75	35	40			51	19	32		
	IV	17	12	5			3	2	1		
Diameter	≤3 cm	270	195	75	21.368 ^b	<0.001 ^a	47	15	32	0.556 ^b	0.456
	>3 cm	81	36	45			102	39	63		

^aP<0.05. ^bZ-value from Kruskal-Wallis H-test. ^cχ² value from Pearson's χ² test. Skp2, S-phase kinase associated protein 2; TNM, tumor-node-metastasis; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; x, lymph node metastasis could not be assessed.

expression in LUAD and LUSC Pearson's χ² was used to compare Skp2 expression in other clinical features, including histological, age, sex, tumor size and distant metastasis. Disease-free survival (DFS) was defined as the period between surgery and the date of recurrence, and overall survival (OS) was considered as the interval from surgery to the date of mortality or final follow-up. Kaplan-Meier method with the log-rank test was performed to determine the difference in survival between groups. Cox proportional hazards regression analysis was performed to estimate the hazard ratios for positive risk factors with the backward conditional elimination method (24). P<0.05 was considered to indicate a statistically significant difference (two sided). SPSS 22.0 statistic software (IBM Corp., Armonk, NY, USA) and GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA) were used to perform the computation for all statistical analyses.

Results

Correlation between Skp2 protein expression assessed by TMA-IHC and clinicopathological parameters in LUSC and LUAD. Skp2 protein expression was assessed by TMA-IHC analysis in the primary lung cancer tissues. Skp2 staining was primarily localized in the nuclear area, which is consistent with a previous study (2) (Fig. 1). Another previous study indicated that Skp2 expression was significantly higher in LUSC, compared with LUAD, which was verified in the present study (P<0.0001; Fig. 1A and B). The median score of Skp2 in LUAD and LUSC were 0.00 and 0.375, respectively. In NSCLC (LUAD and LUSC), the 25th, 50th, 75th quartiles of Skp2 expression final score were 0.000, 0.025 and 0.250. Distribution of the Skp2 expression score in the three intervals were notably different. The majority of Skp2 expression scores

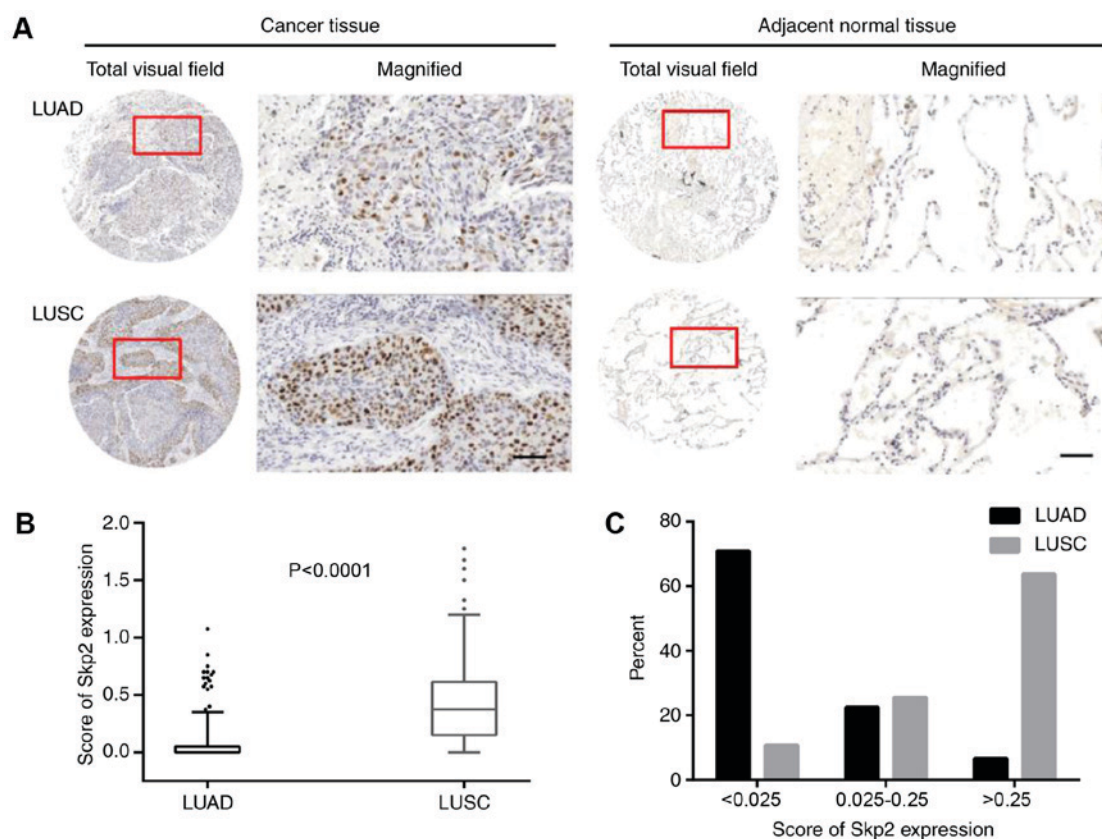


Figure 1. Skp2 expression in lung cancers. (A) Immunohistochemical staining of Skp2 in LUAD, LUSC and adjacent normal tissue (magnification, x100). Red rectangles indicate that the areas been magnified. Scale bars, 100 μm. (B) Boxplot of Skp2 expression scores in LUAD and LUSC. (C) Distribution of Skp2 expression score in the four intervals based on the 25th, 50th and 75th percentile in non-small cell lung cancer. LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; Skp2, S-phase kinase associated protein 2.

were <0.025 in the LUAD group, while scores >0.250 were observed more frequently in LUSC (Fig. 1C). Based on these data, subgroups analysis was proceeded in LUAD and LUSC, separately. A score of 0.025 was used to define high expression and low expression in LUAD, and a score of 0.250 in LUSC was used identically.

In LUAD, Skp2 expression varied in different groups according to sex, differentiation, smoking history, Tumor-Node-Metastasis (TNM) stage (25), lymph node metastasis and tumor diameter ($P < 0.05$; Table I). In LUSC, Skp2 protein expression correlated with smoking status ($P < 0.05$; Table I), whilst it demonstrated limited correlation with other clinical features.

Prognostic significance of Skp2 protein expression in LUAD.

There were three patients who succumbed during the follow-up, shortly following leaving the hospital. Survival analyses were performed in 348 patients with LUAD, of which 120 patients had a high expression of Skp2 and 228 patients had a low expression. Kaplan-Meier survival analysis with log-rank test indicated that a high Skp2 protein expression in patients with LUAD was significantly associated with a reduced DFS and OS ($P < 0.001$; Fig. 2A and B). The 5-year OS in the Skp2-H group was 61.9%, compared with a rate of 86.9% in the Skp2-L group. For patients with LUSC, no association was observed between Skp2 expression and the outcome of patients (Fig. 2C and D).

Cox proportional hazards model was used to determine the association of six factors (sex, age, differentiation, pathological

stage, smoking status and Skp2 protein expression) with the OS of patients with LUAD. As a result, Skp2 protein highly expression was demonstrated to be an independent prognostic factor of OS, with a relative risk of 1.845 ($P = 0.030$). Poor differentiation ($P = 0.016$) and high stage ($P < 0.001$) were associated with a reduced OS also (Table II).

Histological differential mRNAs and frequent mutations association with Skp2. Association analysis and cluster analysis screened nine Skp2 co-expression mRNAs [cell division cycle 45 (CDC45), replication factor C subunit 4 (RFC4), actin like 6A (ACTL6A), family with sequence similarity 83 member D, E2F transcription factor 7, chromosome 15 open reading frame 42 (C15orf42), DNA polymerase θ (POLQ), Skp2 and minichromosome maintenance complex component 2 (MCM2)], which are also differential between LUAD and LUSC. Skp2, CDC45 and MCM2 are involved in the G₁/S transition of the mitotic cell cycle. CDC45, MCM2, RFC4, POLQ and ACTL6A participate in DNA replication and repair (Fig. 3).

In the TCGA cohort of patients with LUAD and LUSC, Skp2 expression was highly expressed in the TP53, NF1 and RB1 mutation groups, compared with the unaltered group. The KRAS mutation group had a lower Skp2 expression in LUAD. Whilst in LUSC, it was determined that there was a significant deviation of Skp2 expression in the four other commonly mutated genes, PTEN, PIK3CA, KEAP1 and NFE2L2 (Table III).

Table II. Multivariate analysis for prognostic factors in patients with lung adenocarcinoma.

Variable	OS				
	Regression coefficient	S.E.	Relative risk	95%CI	P-value
Sex (male vs. female)	-0.221	0.273	0.801	0.469-1.368	0.417
Age (<65 vs. ≥65)	0.536	0.277	1.709	0.993-2.941	0.053
Differentiation (well/moderate vs. poor)	1.282	0.531	3.604	1.272-10.214	0.016 ^a
TNM stage (stage I vs. stages II-IV)	1.484	0.324	4.413	2.340-8.320	<0.001 ^a
Smoking status (non-smoker ^b vs. smoker)	-0.322	0.354	0.752	0.362-1.452	0.364
Skp2 expression (low vs. high)	0.613	0.283	1.845	1.060-3.212	0.030 ^a

^aP<0.05. ^bNever smoked. TNM, tumor-node-metastasis; CI, confidence interval; S.E., standard error; OS, overall survival; Skp2, S-phase kinase associated protein 2.

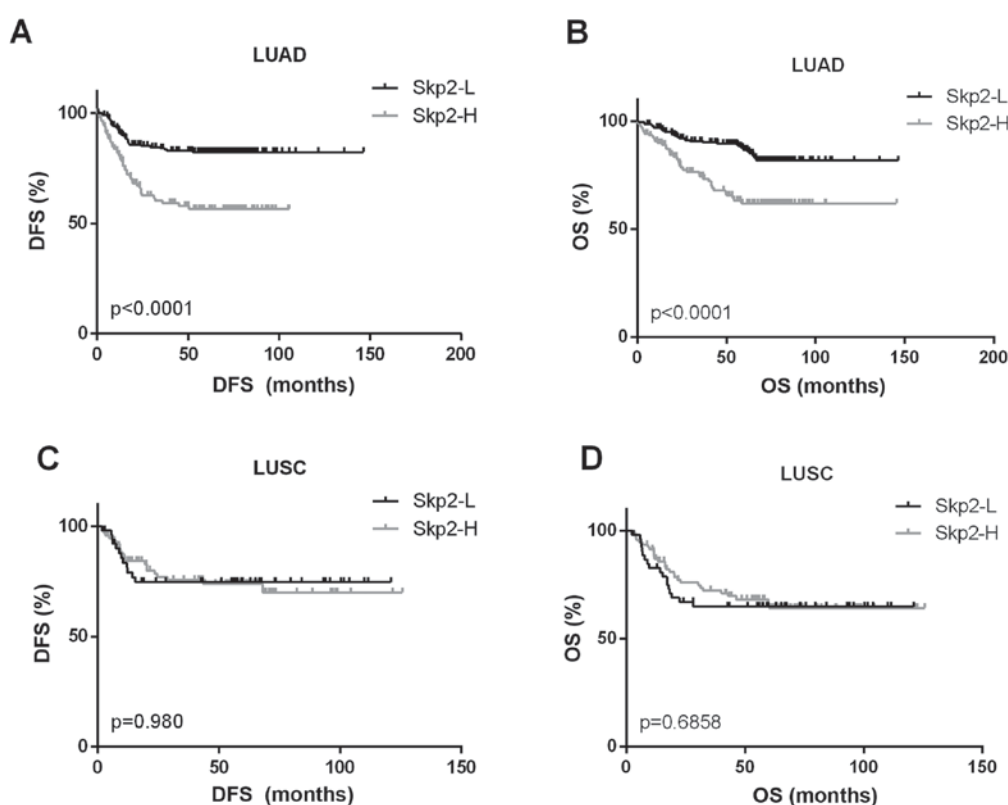


Figure 2. Kaplan-Meier survival analysis for patients with LUAD and LUSC with Skp2-H and Skp2-L. (A) DFS in patients with LUAD. (B) OS in patients with LUAD. (C) DFS in patients with LUSC. (D) OS in patients with LUSC. Skp2-L, Skp2 low expression; Skp2-H, Skp2 high expression; OS, overall survival; DFS, disease-free survival; Skp2, S-phase kinase associated protein 2; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma.

Discussion

LUAD and LUSC are the NSCLC types with the highest prevalence, accounting for 80-85% of lung cancer types. Large-scale sequencing studies have revealed the genomic differences between LUAD and LUSC (26). The molecular mechanisms underlying LUAD and LUSC are considerably different (27). A previous study demonstrated that the Skp2 relative gene copy number aberrations were detected in NSCLC, and these changes are reflected at the mRNA and protein expression levels (16).

In the present study, Skp2 protein has different expression patterns in LUSC and LUAD; therefore the association between

Skp2 expression and clinicopathological parameters in patients with LUAD and LUSC were evaluated. The data indicated that clinicopathological and prognostic implications based on Skp2 expression in LUAD and LUSC should be considered different.

The data indicated that high expression of Skp2 in LUSC would result in abnormal activation of DNA replication and G₁/S transition pathways. This indicated that alterations of lung cancer associated oncogenes modulating these pathways may contribute to the aberration of Skp2 expression. The association between lung cancer mutations and Skp2 expression were then investigated. Skp2 was expressed higher in the lung cancer-associated genes altered group, except in the KRAS

Table III. Deviation of S-phase kinase associated protein 2 expression with lung cancer mutations.

Histology	Gene (n/%)	Mean		SD		P-value	Q-value
		Altered group	Unaltered group	Altered group	Unaltered group		
LUAD	TP53 (111/21.3%)	7.44	7.05	1.08	0.97	9.270x10 ⁻⁴	7.036x10 ⁻³
	KRAS (103/19.7%)	6.90	7.20	0.79	1.04	1.639x10 ⁻³	0.0324
	NF1 (29/13%)	7.61	7.10	1.02	1.00	0.0102	0.193
	RB1 (17/7.4%)	7.98	7.10	1.00	0.99	3.696x10 ⁻⁴	0.0178
LUSC	TP53 (150/29.8%)	9.22	8.79	0.83	0.96	6.32x10 ⁻⁷	6.708x10 ⁻⁶
	PTEN (74/14.7%)	9.24	8.87	0.86	0.95	1.091x10 ⁻³	0.119
	PIK3CA (251/49.8)	9.06	8.77	0.83	1.03	5.430x10 ⁻⁴	2.352x10 ⁻³
	KEAP1 (40/7.9%)	9.28	8.89	0.90	0.94	0.0104	0.0869
	NFE2L2 (58/11.5%)	9.16	8.88	0.88	0.95	7.959x10 ⁻³	0.0348
	RB1 (33/6.5%)	9.39	8.88	0.90	0.94	1.199x10 ⁻³	0.0394

SD, standard deviation; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; TP53, tumor protein P53; NF1, neurofibromin 1; RB1, RB transcriptional corepressor 1; PTEN, phosphatase and tensin homolog; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α ; KEAP1, Kelch-like ECH-associated protein 1; NFE2L2, nuclear factor erythroid 2 like 2.

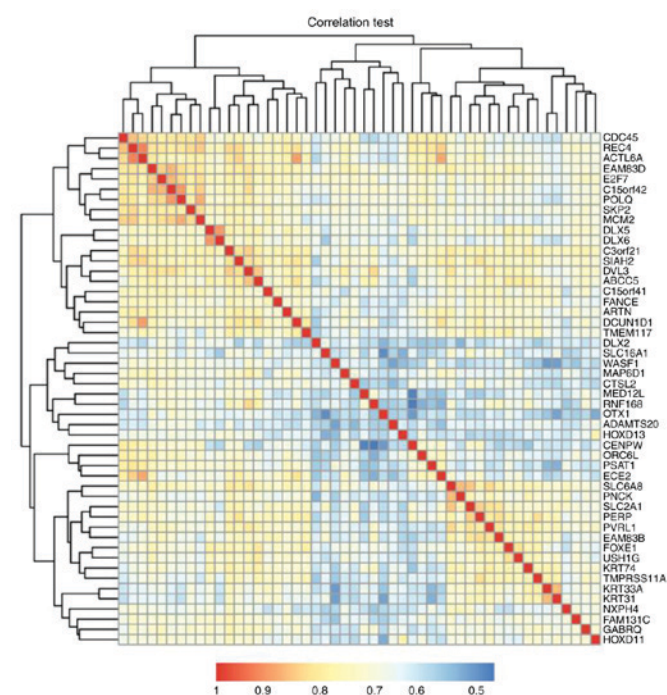


Figure 3. Heatmap with clustering analysis of the top 50 S-phase kinase associated protein 2 co-expressed mRNAs, which were differential between lung adenocarcinoma and lung squamous cell carcinoma.

mutation group. KRAS has a mutation rate of 19.7% in LUAD, which is more frequent than LUSC; however, PTEN, PIK3CA, KEAP1 and NFE2L2, which induce the proliferation of cancer cells, are present more frequently in LUSC. Different driver gene mutation frequencies may be one reason for the distinct expression patterns of Skp2 in LUSC and LUAD; however, this would not fully explain the difference of Skp2 expression between LUAD and LUSC. Further study focusing on the regulation of Skp2 expression are required, including the regulated relationship with deviated miRNAs and lncRNAs.

Skp2 has a function in promoting cell growth (28). A previous study primarily focused on the T stage (29). In the present study, Skp2 overexpression was associated with the tumor diameter more significantly than T stage ($P < 0.001$ vs. $P = 0.221$; Table I). In addition to the T stage, continued studies must also consider pleural invasion and the distance to the carina of the trachea, but these features are not affected by tumor proliferation ability.

The prognostic value of Skp2 expression in NSCLC has been controversial. Osoegawa *et al* (1) indicated that a high Skp2 protein expression was an independent poor prognostic marker in NSCLC; however, Zhu *et al* (30) reported that Skp2 overexpression was not prognostically significant alone, but along with a RAS mutation was a significant independent poor prognostic marker in NSCLC. In the present study, it was demonstrated that a high expression of Skp2 may indicate a poor prognosis in LUAD, but it was not observed in LUSC. Additionally, in KRAS-mutated LUAD, Skp2 had a lower expression level, compared with the unaltered group. This may indicate that Skp2 may have clinicopathological and prognostic implications when it is expressed at a relatively lower level.

Detection of Skp2 overexpression may be a beneficial marker for prognosis. Skp2 may provide sputum-based markers that have the potential to improve the early detection of LUSC (31). Skp2 can also be detected in the peripheral blood of patients with NSCLC, and has demonstrated high diagnostic value (32). These studies provide evidence that detection of Skp2 expression level should be conducted prior to surgery, assisting with the decision for surgery procedure; however, the molecular mechanism underlying regulation of Skp2 in lung cancer remains unclear and further *in vitro* and *in vivo* investigations are required.

To conclude, Skp2 protein expression has different patterns between LUAD and LUSC. The clinicopathological and prognostic implications based on Skp2 expression in LUAD and LUSC should be considered different. Differential expression

of Skp2 protein expression was associated with an unfavorable outcome in patients with LUAD, but not in LUSC. Skp2-HLUSC may have robust proliferation ability.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KZ analyzed and interpreted the data regarding the Skp2 expression and clinicopathological parameters, and also was a major contributor in writing the manuscript. QH and JC performed the tissue microarray and immunohistochemical analyze. FY and JW made substantial contributions to conception and design. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures performed in the current study involving human participants were in accordance with the ethical standards of the Ethics Committee of Peking University People's Hospital and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Consent for publication

Written informed consent for the publication of any associated data and accompanying images was obtained from all the patient, or parent, guardian or next of kin (in case of deceased patients).

Competing interests

The authors declare that they have no competing interests.

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