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ORIGINAL ARTICLE

Phosphatidylinositol 3-kinase could be a promising target in lung cancer therapy

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Summary

Purpose: *To investigate the prevalence of phosphatidylinositol 3-kinase (PIK3CA) gene amplification in lung cancer, and to explore its prognostic value.*

Methods: *A total of 647 lung tumor samples from 290 patients were included in the study. The ratio of PIK3CA signals/centromere 3 signals in cancer cells was estimated by fluorescence in situ hybridization (FISH) analysis.*

Results: *Both gains and amplifications were significantly more frequent in squamous cell (gains: 19.4%; amplifications: 34.1%; p<0.0001) and large cell carcinoma (gains: 22.4%; amplifications: 20.4%; p<0.0001) compared with adenocarcinomas (gains 3.0%; amplifications: 4.0%). Conversely, adenocarcinomas displayed significantly more frequent deletions of the PIK3CA locus than the other two histologic types (p<0.0001). No clear correlation between PIK-3CA status and the pT stage, pN stage or the degree of tumor differentiation was found. Ki67 significantly increased with increasing of PIK3CA copy number: 47 tumors with a PIK-3CA deletion had a mean Ki67 of 16, while 103 tumors with PIK3CA amplification showed a mean Ki67 of 28 (p=0.004). Significant association between cell proliferation and PIK-3CA was found (p<0.05). However, no significant correlation was seen between patient survival and PIK3CA amplifications, deletions and gains.*

Conclusion: *PIK3CA amplifications in large cell and squamous cell carcinomas were significantly higher compared with adenocarcinomas. The results suggest that PIK-3CA could be a promising target for selective lung cancer therapy.*

Key words: amplification, Ki67, lung cancer, phosphatidylinositol 3-kinase

Introduction

Many different genetic aberrations have been detected in lung cancer. Within the large number of these genetic aberrations, mutations of p53 tumor suppressor gene were detected in more than 70% of small cell and in up to 50% of non-small cell lung carcinomas [1]. Tumor-specific changes in gene profiles have been revealed in small cell and non-small cell lung carcinomas by comparative genomic hybridization (CGH) among many chromosomal losses and amplifications [2-5]. Volinia et al. [6] reported that the chromosomal localization of the PIK3CA gene was at 3q26, as determined using somatic cell hybrids.

PIK3 plays an important role in PIK3/Akt-signaling pathway, which is regarded as one of the most frequently activated signaling pathways in malignant human tumors in general [7]. PIK3 phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3), thereby allowing the binding of proteins that have the same proviso "pleckstrin homology domain" to PIP3. These proteins are recruited to the cell membrane where they can then be activated by binding to PIP3. An important representative, PIK3, is also involved in other signaling pathways during cell growth, proliferation, migration, and metabolism, which regulate protein synthesis and degradation. The tumor suppressor PTEN acts as an antagonist of PIK3 by dephosphorylating PIP3 to PIP2 [8,9].

Alterations of the PIK3CA gene were found in many human malignancies. Amplifications were found in breast (9%), endometrial (10%), cervix (9%), ovarian (12%), esophagus (6%), stomach (36%)) and thyroid carcinomas (9%), glioblastoma (6%) and in head and neck tumors (32%) [10-14]. In lung cancer, PIK3CA amplification was found in 33-61% of squamous cell carcinoma, 0-17% of adenocarcinoma, 17-25% of large cell carcinoma and 17-50% of small cell carcinomas [15-18]. PIK3CA mutations were found in 1.6-3.4% of non-small

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cell lung carcinomas [15-19]. Shibata et al. found activating mutations of PIK3CA in 13% of small cell lung carcinomas [20].

PIK3CA alterations increased the PIK3 activity and the level of activated Akt, and were associated with tumor growth. The mutations of the PIK3CA were expressed by raised siRNA and shRNA in lung carcinoma cell lines with amplifications and mutations of PIK3CA [18]. Treatment with the PIK3 inhibitor "LY294002" resulted in gefitinib resistance in non-small cell lung carcinoma cell lines [21]. PI-103 as a PIK3 inhibitor showed marked antitumor activity in gefitinib-resistant non-small cell lung cancer cells [22]. This illustrated the relevance of PIK3CA to the survival of tumor cells and made PIK3/Akt-signaling pathway a promising target site in cancer therapy. Therefore, the aim of this study was to investigate the prevalence of PIK3CA amplification in lung cancers and its prognostic relevance.

Methods

Tumor samples

A total of 647 lung tumor samples were included in the study. The samples were obtained from 290 patients with median age of 62 years (range 5-92). Survival data were collected from the medical records of patients treated at the Nanfang hospital of Southern Medical University. The median follow-up time was 29 months (range 4-48). The tissue sections of each sample were independently evaluated and registered by two pathologists at Nanfang hospital. The extent of the primary tumor (pT), the pathologic nodal status (pN) and the grade of tumor differentiation as well as tumor histology were the end points of histological analysis.

Fluorescence in situ hybridization (FISH)

For the two-color FISH analysis, 4 micro thick sections were used. Prior to hybridization, the sections were treated in VP 2000 Pretreatment Reagent (Vysis, USA) at 80° C for 15 min, deparaffinized and incubated at 37° C for 150 min in VP 2000 protease buffer (0.01 N Hcl; Vysis, USA) according to the protocol of the Paraffin Pretreatment Reagent Kit (Vysis, USA). For hybridization, a self-produced PIK3CA digoxigenierte BAC probe (BAC RP11-245C23, RZPD, Germany) was used, which included the complete PIK3CA gene. The marking of the self-prepared DNA probe was performed by nick translation using Nick Translation System (Invitrogen, USA). For reference, a commercial probe for the centromere of chromosome 3 (Spectrum orange, Vysis) was also used. The commercial probe was not included in the diluted hybridization mixture. Four µl of gene-specific probe and the reference probe were mixed with 2 µl human Cot-DNA (to block nonspecific binding sites or repetitive sequences) and this hybridization mixture was applied to the sections for 10 min at 72° C and incubated overnight at 37° C. Following hybridization, the sections were washed stringently to remove non-specific hybridizations. The detection of the hybridized sections was carried out with Fluorescent Antibody Enhancer Set (Roche, Switzerland).

FISH analysis

To ensure a rapid evaluation of the samples, the ratio of PIK-3CA signals/centromere 3 signals in the cancer cells was estimated for each tissue sample. Amplification was defined as at least twice as many signals as the gene-specific probe signals from the reference probe (PIK3CA/Cen3 ratio $\geq$ 2.0). Tissue samples with PIK3CA/Cen3 ratio of 1.0-2.0 were designated as gains, with PIK3CA/Cen3 ratio 1.0 as normal and with PIK3CA/cen3 ratio <1.0 as deletion.

Statistical considerations

The statistical analysis was performed using the JMP software (SAS Institute Inc., USA). The chi-square test was used to compare the PIK3CA amplification with the clinicopathological variables and p53 status. Analysis of variance (ANOVA test) was performed for the PIK3CA status with cell proliferation. Kaplan-Meier analysis was used to test the correlation of PIK3CA and patients' survival. Log-rank test was used to test survival differences among categorical variables and a p-value < 0.05 was considered as the level of statistical significance.

Results

Details of pT and pN stage, grade of differentiation and histologic subtypes of tumor samples are shown in Table 1, and Figure 1 shows representative

Table 1. Primary tumor pathological status (pT), pathological nodal status (pN), grade of tumor differentiation (G) and histological subtypes

		N	*%*
All samples		616	100.0
pT	1	185	30.0
	2	320	52.0
	3	51	8.3
	4	53	8.6
	Unknown	7	1.1
pN	0	266	43.2
	1	162	26.3
	2	108	17.5
	3	22	3.6
	Unknown	58	9.4
G	1	16	2.6
	2	339	55.0
	3	187	30.4
	4	41	6.6
	Unknown	33	5.4
Histological subtype	SCC	247	40.1
	ADC	210	34.1
	LCC	105	17.0
	SCLC	19	3.1
	LCNEC	15	2.4
	BAC	12	2.0
	cSCLC	5	0.8
	cNSCLC	2	0.3
	cNSCLC	2	0.3

pT: primary tumor, pN: pathologic nodal status, SCC: squamous cell carcinoma, ADC: adenocarcinomas, LCC: large cell carcinoma, SCLC: small cell lung cancer, LCNEC: large cell neuroendocrine carcinoma, BAC: bronchioloalveolar carcinoma, cSCLC: combined small cell lung cancer, cNSCLC: combined non small cell lung cancer

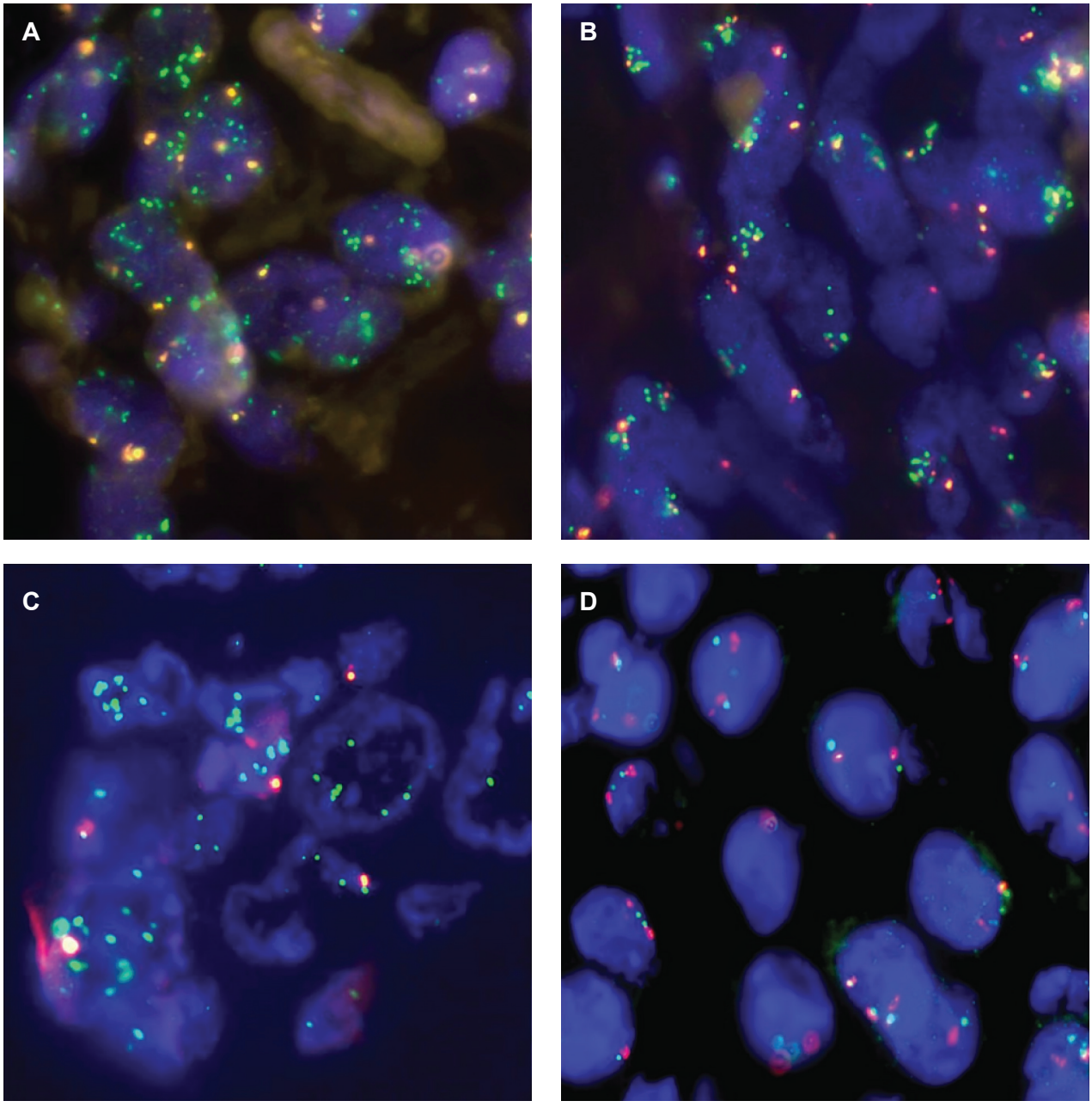

Figure 1. Examples of PIK3CA FISH findings. **A-C:** amplifications; **A)** 6-15 green (PIK3CA) and 1-2 orange (centromere 3) in large cell lung carcinoma; **B)** 20-25 and 2-10 green orange in lung squamous cell carcinoma; **C)** 7-10 green and an orange in lung adenocarcinoma; **D)** normal findings (2 green, 2 orange) in lung squamous cell carcinoma.

photomicrographs of PIK3CA FISH findings in different histological subtypes of lung tumors.

In 581 analyzable samples amplifications were observed in 18.8% and gains in 12.7%. In addition, relative PIK3CA loss (less than gene-centromere signals) was found in 8.8% of the tumors. Tumors with amplifications showed an average of 9.9 gene copies, with high-level amplifications (10 or more copies of genes) in nearly half (48.6%) of all amplified tumors.

Both gains and amplifications were more frequent in squamous cell and large cell carcinomas compared with adenocarcinomas, and the differences were statistically significant: gains for squamous cell and adenocarcinoma: 19.4 vs. 3.0%; $p<0.0001$; gains for large cell carcinoma and adenocarcinoma: 22.4 vs. 3.0%; $p<0.0001$; amplifications for squamous cell and adenocarcinoma: 34.1 vs. 4.0%, $p<0.0001$; amplifications for large cell carcinoma and adenocarcinoma: 20.4 vs. 4.0%; $p<0.0001$. Conversely, deletions of the PIK3CA locus were significantly more frequent in adenocarcinomas than in the other two histologic types ($p <0.0001$).

No clear correlation between PIK3CA status and

the pT stage, pN stage or the grade of tumor differentiation was found. No obvious proportional increase or decrease was observed in the PIK3CA copy number with increasing pT, pN, or grade of tumor differentiation, suggesting a relevant biological context. All results are summarized in Table 2.

The cell proliferation rates were analyzed in a total of 523 tumor samples according to Ki67 and PIK3CA, respectively. Comparison showed that Ki67 significantly increased with increasing PIK3CA copy number. Forty-seven tumor samples with a PIK3CA deletion had a mean Ki67 of 16, while 103 tumor samples with amplification showed a mean Ki67 of 28 (p = 0.004, Figure 2A). The association between cell proliferation and PIK3CA was also significant when only the pure gene copy number was considered (Figure 2B).

Table 2. Association between tumor phenotype and PIK3CA

	Total	*Analyzed (%)*	*FISH PIK3CA*				*p-value*
			Del (%)	*Norm (%)*	*Gain (%)*	*AMP (%)*	
All samples Histologies	647	581 (89.8)	51 (8.8)	347 (59.7)	74 (12.7)	109 (18.8)	
SCC	247	232 (93.9)	4 (1.7)	104 (44.8)	45 (19.4)	79 (34.1)	<0.0001*
ADC	210	199 (94.8)	31 (15.6)	154 (77.4)	6 (3.0)	8 (4.0)	<0.00145**
LCC	105	98 (93.3)	5 (5.1)	51 (52.0)	22 (22.4)	20 (20.4)	<0.0001*
SCLC[§]	19	18 (94.7)	5 (27.8)	11 (61.1)	1 (5.6)	1 (5.6)	
LCNEC[§]	15	15 (100.0)	4 (26.7)	10 (66.7)	0 (0.0)	1 (6.7)	
BAC[§]	12	12 (100.0)	1 (8.3)	11 (91.7)	0 (0.0)	0 (0.0)	
cSCLC[§]	5	4 (80.0)	1 (25.0)	3 (75.0)	0 (0.0)	0 (0.0)	
cNSCLC[§]	2	2 (100.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	
pT							0.0034
1	185	167 (90.3)	19 (11.4)	117 (70.1)	11 (6.6)	20 (12.0)	
2	320	312 (97.5)	20 (6.4)	177 (56.7)	49 (15.7)	66 (21.2)	
3	51	49 (96.1)	5 (10.2)	25 (51.0)	5 (10.2)	14 (28.6)	
4	53	47 (88.7)	6 (12.8)	26 (55.3)	7 (14.9)	8 (17.0)	
pN							0.0043***
0	266	254 (95.5)	23 (9.1)	166 (65.4)	28 (11.0)	37 (14.6)	
1	162	155 (95.7)	10 (6.5)	77 (49.7)	26 (16.8)	42 (27.1)	
2	108	103 (95.4)	13 (12.6)	53 (51.5)	14 (13.6)	23 (22.3)	
3	22	21 (95.5)	0 (0.0)	16 (76.2)	3 (14.3)	2 (9.5)	
G							0.0042
1-2	355	337 (94.9)	24 (7.1)	193 (57.3)	39 (11.6)	81 (24.0)	
3-4	228	213 (93.4)	23 (10.8)	129 (60.6)	34 (16.0)	27 (12.7)	

*SCC vs. ADC; ** SCC vs. LCC; *** p=0.0086, pN0 vs. pN (1-3)
For abbreviations see footnote of Table 1. [§]No p-value due to small sample size

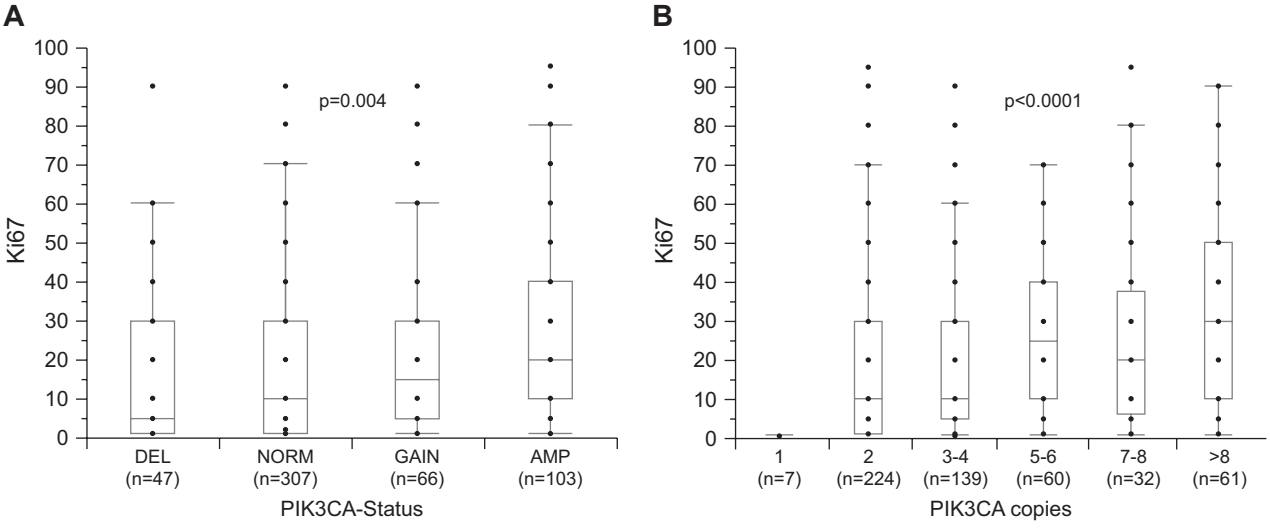

Figure 2. A: Correlation between PIK3CA status and proliferation rate. **B:** Correlation between PIK3CA copy number and proliferation rate.

PIK3CA status was determined in 263 patients with clinical history data. However, no significant correlation was seen between patient survival and PIK3CA amplifications, deletions or gains (data not shown).

Discussion

In the present study, more than 600 lung cancer tissue samples were tested with an aim to clarify the significance of PIK3CA gene amplification. The results showed that PIK3CA amplification occurred frequently in lung cancer, and the level of amplification differed significantly among different histological subtypes. These results not only support the hypothesis that squamous cell carcinoma and adenocarcinoma might have a different mechanism of tumorigenesis [4], but also suggest that large cell carcinoma might also arise in another way as adenocarcinomas. In a study, PIK3CA amplification rate ranged between 18 and 33% [15].

However, adenocarcinomas showed frequent (16%) deletions of the PIK3CA locus. This result fitted well with the results of a study by Petersen et al. [2], but the comparison of 25 adenocarcinomas and 25 squamous cell carcinomas were significantly more frequent in adeno-3q deletions (36%) than in squamous cell carcinomas (20%). The findings therefore supported the hypothesis of a different tumorigenesis. Theoretically, it would also be conceivable that the PIK3CA loss in these tumors was not biologically relevant, if simultaneous inactivation of the direct molecular antagonists of PIK-3CA were present. Although the rather rare genomic PTEN aberrations (<5%) could be considered as a cause of non-small cell lung cancer, downregulation of PTEN protein was frequently observed (34-74%) in non-small cell lung carcinomas, and this downregulation by promoter hypermethylation might be associated with an epigenetic silencing of the PTEN gene [25]. However, no clear evidence supported that PTEN loss might occur more frequently in adenocarcinomas, so a possible coincidence of PTEN inactivation and PIK3CA loss is largely speculative. Small cell carcinomas are considered to be particularly aggressive and this may be due to the relatively rare PIK3CA amplifications (5.6%).

Our research revealed that there is no association between PIK3CA overexpression and primary tumor size or nodal status, in agreement with 2 previous studies [16,26]. This indicated that PIK3CA probably exerted no biological role in tumor progression. It could therefore be a rather relatively early change that may be associated with tumor initiation. A previous study showed an early role of PIK3CA amplification in the transition from low-grade to high-grade dysplasia to invasive carcinoma in head and neck squamous dysplasia and squamous cell carcinoma [27]. Thus increased gene copy numbers in 3q26 were found in 17% of the cases with low-to-moderate dysplasia, in 78% of high-grade dysplasia and in 100% of cancers. The increased copy numbers also increased the expression of p110α mRNA and protein. Massion et al. also found an increased PIK3CA copy number in the moderate to high grade squamous dysplasia observed in normal mucosa, while only normal copy numbers were observed in mild dysplasia [26].

In view of the generally poor prognosis of lung cancer patients, it is not surprising that no effect of PIK3CA on the survival of lung cancer patients was detected in this study. However, Kawano et al. found a lower survival rate in patients with PIK3CA-amplified lung cancer. The results of this study showed a strong association with cell proliferation measured by Ki67. This showed that tumors with PIK3CA deletion or a few gene copies had the lowest rate of proliferation, whereas high grade tumors or with high copy numbers had PIK3CA amplifications. This finding matches well with the direct influence of PIK3CA on PIK3/Akt-signaling pathways. This pathway regulated cell growth, proliferation, migration, angiogenesis and apoptosis [28]. Some studies showed that the PIK3CA expression upregulated PIK3CA amplifications, and thus increased the PI3K activity [15,17,27].

In summary, PIK3CA amplifications are common in lung cancers, particularly in squamous cell and large cell carcinoma. Apparently, it is a early event occurring in tumor development, because PIK3CA amplification was involved in pT, pN and grade of tumor differentiation. The presence of a PIK3CA amplification is associated with high tumor proliferation rate. This indicates that the incidence of PIK3CA amplifications in large cell and squamous cell carcinomas was significantly higher adenocarcinomas. The results suggested that PIK3CA could be a promising target for targeted cancer therapy.

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