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Original contribution

Correlation between immunohistochemistry and *RICTOR* fluorescence in situ hybridization amplification in small cell lung carcinoma☆,☆☆,★

Ildiko Krencz MD[a], Anna Sebestyen PhD, MSc[a], Judit Papay MD PhD[a], Yanyan Lou MD PhD[b], Gabrielle F. Lutz BA[d], Tracy L. Majewicz BS[c], Andras Khoor MD PhD[c,*]

[a]*1st Department of Pathology and Experimental Cancer Research, Semmelweis University, Budapest, Hungary H-1085*
[b]*Division of Hematology and Medical Oncology, Mayo Clinic, Jacksonville, FL 32224*
[c]*Department of Laboratory Medicine & Pathology, Mayo Clinic, Jacksonville, FL 32224*
[d]*Clinical Research Internship Study Program, Mayo Clinic, Jacksonville, FL 32224*

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Summary Small cell lung carcinoma (SCLC) accounts for approximately 15% of all lung cancers and remains a challenging disease, with no significant improvement in the field of targeted therapies. The *RICTOR* gene (rapamycin-insensitive companion of mTOR [mammalian target of rapamycin]), which encodes a key structural (scaffold) protein of mTOR complex 2), has recently been identified as one of the most frequently amplified genes and a potential therapeutic target in SCLC. The aim of this study was to compare immunohistochemical (IHC) expression of Rictor and phospho-Akt (a downstream target of mTOR complex 2) with *RICTOR* amplification as detected by fluorescence in situ hybridization (FISH) in SCLC. *RICTOR* FISH and Rictor and phospho-Akt IHC staining were performed on 100 formalin-fixed, paraffin-embedded SCLC samples. *RICTOR* amplification was detected in 15 samples (15%). IHC positivity for Rictor and phospho-Akt was observed in 37 (37%) and 42 (42%) samples, respectively. Considering FISH as the diagnostic standard, the sensitivity and specificity of Rictor IHC were 93% and 73%, whereas the sensitivity and specificity of phospho-Akt IHC were 80% and 65%, respectively. Rictor expression was higher in distant metastases than in primary tumor samples and lymph node metastases. There was no association between *RICTOR* amplification and clinical outcome. However, high expression of either Rictor or phospho-Akt was associated with significantly decreased overall survival. In conclusion, IHC expression of Rictor correlates highly

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★ Reprints: Andras Khoor, MD, PhD, Department of Laboratory Medicine & Pathology, Mayo Clinic, 4500 San Pablo Rd, Jacksonville, FL 32224.
* Corresponding author.
E-mail address: Khoor.Andras@mayo.edu (A. Khoor).

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with *RICTOR* amplification. Therefore, Rictor IHC can be used as a cost-effective method to select patients for *RICTOR* FISH and, potentially, for mTORC1/2 inhibitor therapy.

1. Introduction

Small cell lung carcinoma (SCLC) is an aggressive tumor accounting for approximately 15% of all lung cancers [1]. In most patients, the disease is at an advanced stage at diagnosis because of rapid tumor growth and early metastatic dissemination. Despite extensive research over the past several decades, SCLC remains a challenging disease to treat, with no significant improvements in targeted therapies [2]. In most cases, the treatment remains platinum-based chemotherapy [3,4].

Genomic instability in SCLC is higher than in most other cancers. Genetic alterations frequently involve genes related to cell cycle regulation and the PI3K/AKT/mTOR (mammalian target of rapamycin) pathway [3]. Moreover, *RICTOR* (rapamycin-insensitive companion of mTOR) has recently been identified as the most frequently amplified gene in SCLC [4-8].

mTOR forms 2 multiprotein complexes: mTORC1 (mTOR complex 1), which contains the scaffold protein Raptor, and mTORC2 (mTOR complex 2), which contains the scaffold protein Rictor. Activation of mTORC1 leads to phosphorylation of eukaryotic initiation factor 4E–binding protein 1, S6 kinase, and subsequently ribosomal S6 protein, which facilitates protein translation, cell growth, and cell proliferation. Activation of mTORC2 leads to phosphorylation of Akt at Ser473, which is implicated in cell survival and cytoskeleton remodeling [9,10].

mTORC1 and mTORC2 have important roles in the pathobiology of many neoplasms, including adenocarcinoma of the lung and lymphangioleiomyomatosis [11,12]. The utility of mTORC1 inhibitors has been established in lymphangioleiomyomatosis. However, they are less effective in other types of lung cancer, most likely because of their limited single-agent activity and a lack of predictive biomarkers for tumor selection [13]. For example, everolimus, an mTORC1 inhibitor, has shown limited single-agent activity in unselected, previously treated patients with SCLC [14].

Fewer studies have investigated the effectiveness of mTORC1/2 inhibitors in cancer therapy. However, some of these studies suggest that amplification of *RICTOR* may predict a response to mTORC1/2 inhibitors [4,15]. More recently, vistusertib, an mTORC1/2 inhibitor, has been investigated in phase II clinical trials for its effect on *RICTOR*-amplified and unselected tumors [16].

Immunohistochemical (IHC) analysis of mTORC2-related proteins could be used similarly to fluorescence in situ hybridization (FISH) analysis of *RICTOR* for patient selection. IHC is also more widely available and less expensive, and has a shorter turnaround time than FISH [15,17]. Despite these potential advantages, expression of mTORC2-related proteins has not previously been evaluated in SCLC. The aim of this study was to compare IHC expression of Rictor and phospho-Akt (a downstream target of mTORC2 and a widely accepted marker for mTORC2 activation) with *RICTOR* amplification as detected by FISH in SCLC. We aimed to determine whether IHC for Rictor may serve as a useful screening method to select tumors for further evaluation by *RICTOR* FISH.

2. Materials and methods

2.1. Case selection

The study was approved by the Mayo Clinic Institutional Review Board. We searched our pathology database for the records of patients with SCLC between August 1, 2008, and April 30, 2018, at Mayo Clinic, Jacksonville, FL, who had tumor biopsy samples available. For patients who met these inclusion criteria, samples were retrieved for study. The samples were re-reviewed and tumors reclassified according to the "2015 World Health Organization Classification of Lung Tumors" [18]. The diagnosis was supported by IHC for cytokeratin, AE1/AE3, TTF-1, synaptophysin, and chromogranin in most cases. No patients received chemotherapy before the biopsy. Cell blocks with at least 500 tumor cells were analyzed.

2.2. *RICTOR* FISH

Sections of formalin-fixed, paraffin-embedded tissue blocks 4 μm thick were used for FISH analysis. After deparaffinization and pretreatment at 79°C for 25 minutes in Vysis IntelliFISH Pretreatment SSC Solution (Abbott Molecular, Abbott Park, IL, USA), protease digestion was performed at 38°C for 25 minutes (Vysis IntelliFISH Protease; Abbott Molecular). Probes for *RICTOR* (#RICTOR-20-OR; Empire Genomics, Williamsville, NY, USA) and chromosome 5 (Chr5) control (#CHR05-10-GR; Empire Genomics) were mixed with hybridization buffer (Vysis IntelliFISH Hybridization Buffer; Abbott Molecular) and hybridized at 37°C for 2 hours. After a posthybridization wash at 73°C for 3 minutes (Vysis IntelliFISH Post-Hybridization Buffer; Abbott Molecular) and air drying, sections were counterstained with DAPI I (Abbott Molecular).

2.3. FISH interpretation

Areas of slides with sufficient neoplastic cells were marked by a pulmonary pathologist (A. K.). Slides were examined under a fluorescence microscope (DM5500 B; Leica, Buffalo Grove, IL, USA), and images were captured using CytoVision software (Leica). Focusing on hot spots, 2 technicians individually evaluated 30 tumor cell nuclei from at least 2 different areas and counted the orange *RICTOR* and green Chr5 signals. For each tumor, the mean number of signals per nucleus was determined, and the *RICTOR*/Chr5 control ratio was established. A *RICTOR* copy number of less than 4 with a *RICTOR*/Chr5 ratio of less than 2 was considered *negative*. A *RICTOR* copy number of 6 or more or a *RICTOR*/Chr5 ratio of 2 or more was considered *positive*. A *RICTOR* copy number of 4 or more to less than 6 with a *RICTOR*/Chr5 ratio of less than 2 was considered *equivocal*.

2.4. Immunohistochemistry

IHC staining for Rictor and phospho-Akt was performed on 4-μm–thick sections. After deparaffinization and endogenous peroxidase blocking, antigen retrieval was performed for 30 minutes (10 mmol/L citrate, pH 6.0) using a pressure cooker. Slides were incubated with anti-Rictor (1:1000; #A500-002A; Bethyl Laboratories, Inc, Montgomery, TX, USA) and anti–phospho(Ser473)-Akt (1:100; cat. #4060; Cell Signaling Technology, Inc, Danvers, MA, USA) primary antibodies. Vectastain Universal Elite ABC HRP Kit (Vector Laboratories, Burlingame, CA, USA) and Novolink Polymer (Leica Biosystems) detection systems were used for the anti-Rictor and anti–phospho(Ser473)-Akt antibodies, respectively. DAB (Aligent; Santa Clara, CA, USA) was used as chromogen, and sections were counterstained with hematoxylin.

2.5. IHC scoring

Immunostained sections were reviewed independently by 2 investigators (J. P. and I. K.). In each case, they determined the percentage of positive cells of any intensity by evaluating 500 cells in hot spots. To make protein expression levels more comparable, cutoff values were determined for each antibody based on the distribution of data. The 15th, 50th, and 85th percentiles of positive cells were used as starting points and rounded to the nearest integer divisible by 5 to establish cutoff values between no and low expression, low and moderate expression, and moderate and high expression, respectively. Rictor IHC staining was interpreted as follows: no expression, staining 5% or less of tumor cells; low expression, staining more than 5% to 20% or less of tumor cells; moderate expression, staining more than 20% to 50% or less of tumor cells; high expression, staining more than 50% of tumor cells. Phospho-Akt IHC staining was interpreted as follows: no expression, staining 5% or less of tumor cells; low expression, staining more than 5% to 35% or less of tumor cells; moderate expression, staining more than 35% to 70% or less of tumor cells; high expression, staining more than 70% of tumor cells. No expression and low expression were defined as *negative*, whereas moderate and high expression were defined as *positive*, for both Rictor and phospho-Akt staining. For archival purposes, images of the IHC stains were captured using Pannoramic 1000 Digital Slide Scanner (3DHistech Ltd, Budapest, Hungary) and CaseViewer 2.2 software (3DHistech Ltd).

2.6. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics software, version 22 (SPSS Inc, Chicago, IL). Descriptive data were expressed as number (percentage) or median (range). By using a dichotomous evaluation method (negative versus positive), sensitivity and specificity of IHC were evaluated, with FISH as the diagnostic standard. Spearman rank correlation was used to assess correlation between *RICTOR* copy number and Rictor and phospho-Akt expression. The Fisher exact test was used to determine associations between clinicopathological parameters and *RICTOR* amplification, and Rictor and phospho-Akt expression. The Kaplan-Meier method was used to estimate survival, and the log-rank method was used to compare survival curves. Statistical significance was defined as $P \leq .05$.

3. Results

A total of 100 samples were obtained from 92 patients during the study period. Six patients had 2 different samples and 1 patient had 3 different samples from various collection dates (each of the remaining patients had 1 sample). Clinicopathological data are summarized in Table 1.

3.1. *RICTOR* amplification in SCLC

RICTOR amplification was assessed using FISH in all 100 SCLC samples (Fig. 1A and B). The median (range) *RICTOR* copy number was 2.90 (1.26-8.35) in the tumor cells. *RICTOR* amplification was observed in 15 of the 100 cases analyzed (15%). Of the remaining 85 cases, 3 cases (3%) were equivocal and 82 cases (82%) were negative for *RICTOR* amplification (Table 2).

3.2. Rictor and phospho-Akt expression in SCLC

Expression of Rictor (a key structural component of mTORC2) (Fig. 1C and D) and phospho-Akt (a downstream target of mTORC2 and a marker for its activation) (Fig. 1E and F) was analyzed by IHC. Rictor positivity was detected in 37 of 100 cases (37%). Among these 37 cases, 14 (14%) showed high Rictor expression and 23 (23%) showed

Table 1 Clinicopathological data

Variable	Patients (n = 92)	Samples (n = 100)
Age, y		
<65	29 (32)	
≥65	63 (68)	
Men	54 (59)	
Tumor type		
Primary tumor		30 (30)
Lymph node metastasis		52 (52)
Distant metastasis		18 (18)
Procedure		
Fine needle aspiration biopsy (cell block)		75 (75)
Bronchial brushing/washing (cell block)		4 (4)
Thoracocentesis (cell block)		1 (1)
Transbronchial biopsy		4 (4)
Wedge biopsy		3 (3)
Lobectomy		7 (7)
Excision biopsy of the distant metastasis		6 (6)

NOTE. Values are number of patients or samples (%).

moderate Rictor expression (Table 2). Phospho-Akt positivity was detected in 42 cases (42%): 16 (16%) with high phospho-Akt expression and 26 (26%) with moderate phospho-Akt expression.

3.3. Correlation between *RICTOR* FISH and Rictor and phospho-Akt IHC

RICTOR copy number showed positive correlation with both Rictor ($\rho = 0.416$; $P < .001$) and phospho-Akt ($\rho = 0.289$; $P < .01$) expression. A strong positive correlation was also detected between Rictor and phospho-Akt protein expression ($\rho = 0.466$; $P < .001$).

Of the 15 cases in which *RICTOR* was amplified, 14 (93%) were positive for Rictor (5 high expression, 9 moderate expression), and 12 (80%) were positive for phospho-Akt (5 high expression, 7 moderate expression) (Fig. 2). One *RICTOR*-amplified case was negative for both Rictor and phospho-Akt. In contrast, among the 85 nonamplified cases (negative or equivocal for *RICTOR* amplification), 23 (27%) were

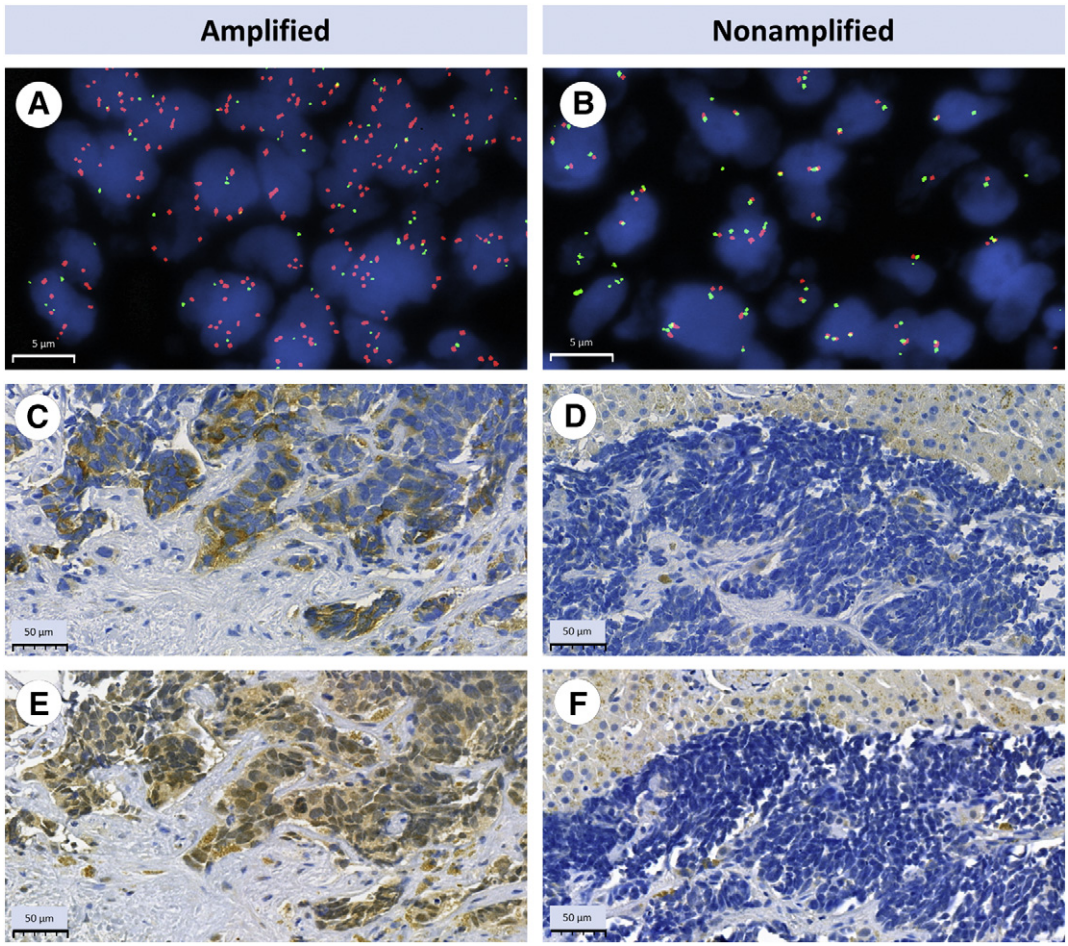

Figure 1 Examples of *RICTOR* FISH and Rictor and Phospho-Akt IHC in SCLC samples. *RICTOR* FISH showing amplification (A) and non-amplification (B). IHC for Rictor showing high expression (C) and no expression (D). IHC for phospho-Akt showing high expression (E) and no expression (F). D and F, Peritumoral liver tissue was positive for either Rictor or phospho-Akt and was used as an internal positive control.

Table 2 *RICTOR* amplification detected by FISH and IHC positivity for Rictor and phospho-Akt in SCLC samples

Test findings	All samples (N = 100)	Tumor type			*P*
		Primary tumor (n = 30)	Lymph node metastasis (n = 52)	Distant metastasis (n = 18)	
RICTOR FISH					.34
Positive	15	2	8	5	
Equivocal	3	1	2	0	
Negative	82	27	42	13	
Rictor expression by IHC					<.001
High	14	0	5	9	
Moderate	23	7	12	4	
Low	25	9	12	4	
None	38	14	23	1	
Phospho-Akt expression by IHC					.09
High	16	2	7	7	
Moderate	26	7	16	3	
Low	35	10	20	5	
None	23	11	9	3	

NOTE. Values are number of samples.

positive for Rictor (9 high expression, 14 moderate expression), and 30 (35%) were positive for phospho-Akt (11 high expression, 19 moderate expression). In 19 of the 23 nonamplified cases (83%) with Rictor positivity, phospho-Akt positivity was also detected.

With *RICTOR* amplification as detected by FISH considered the diagnostic standard, the sensitivity and specificity of Rictor IHC were 93% and 73%, respectively. In contrast, the sensitivity and specificity of phospho-Akt IHC were 80% and 65%, respectively (Table 3).

3.4. Association between *RICTOR* amplification, Rictor and phospho-Akt expression, and clinicopathological parameters

There was no association between the presence or absence of *RICTOR* gene amplification and clinicopathological parameters such as age, sex, tumor type (primary versus metastasis), and procedure (data not shown). Interestingly, Rictor expression was significantly higher in distant metastases than in primary tumors and lymph node metastases ($P < .001$) (Table 2). Phospho-Akt expression was also higher in distant metastases, but these results were not statistically significant ($P = .09$). There was no association between Rictor and phospho-Akt expression and age, sex, and procedure (data not shown).

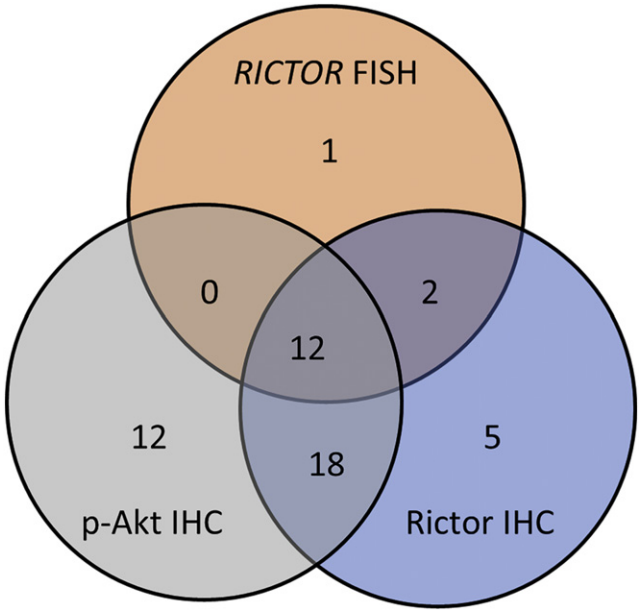

Figure 2 Venn diagram illustrating concordance between *RICTOR* FISH, Rictor IHC, and p-Akt IHC. The diagram shows the number of positive cases for each method.

3.5. Association between *RICTOR* amplification, Rictor and phospho-Akt Expression, and overall survival

There was no association between *RICTOR* amplification determined by FISH and overall survival (Fig. 3A). In contrast, high expression of either Rictor (log-rank $P = .007$) or phospho-Akt (log-rank $P < .001$) was associated with significantly decreased survival compared with no expression of the same marker (Fig. 3B and C).

4. Discussion

In this study, we analyzed the presence of *RICTOR* amplification, as well as Rictor and phospho-Akt expression, in SCLC. In prior studies, the prevalence of *RICTOR* gene alterations in SCLC has varied from 6% to 14% [4-6,8,16]. The 15% frequency of *RICTOR* amplification in our study was

Table 3 Concordance of *RICTOR* FISH and IHC results

	RICTOR FISH	
	Amplified	Nonamplified [a]
Rictor expression		
Positive	14 (Sensitivity = 93%)	23
Negative	1	62 (Specificity = 73%)
Phospho-Akt expression		
Positive	12 (Sensitivity = 80%)	30
Negative	3	55 (Specificity = 65%)

[a] Negative or equivocal for *RICTOR* amplification.

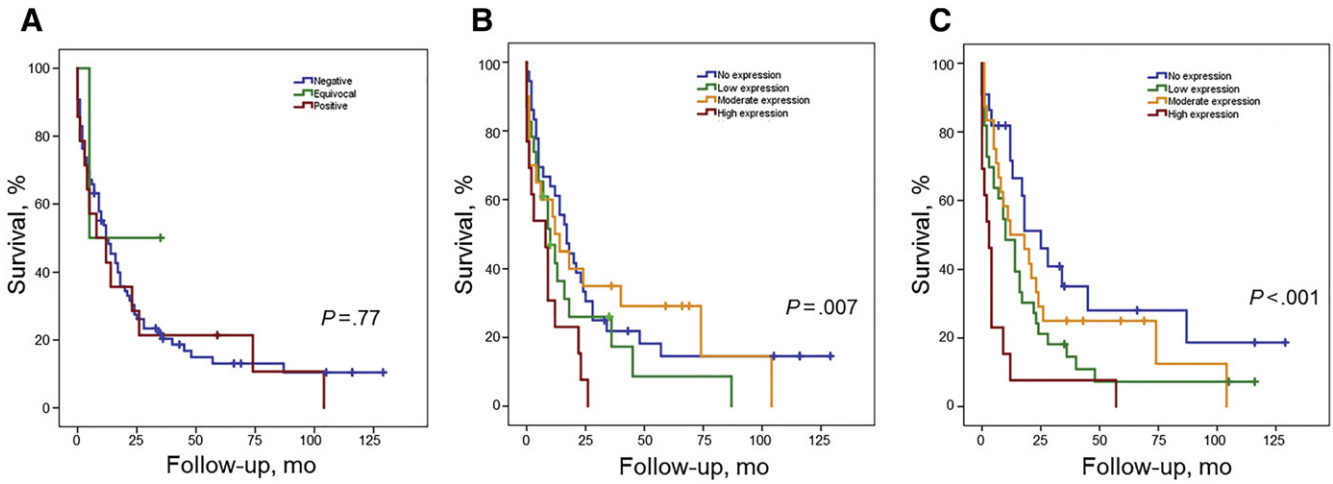

Figure 3 The effect of *RICTOR* amplification and expression of Rictor and phospho-Akt proteins on overall survival of patients with SCLC. Kaplan-Meier survival curves for association between survival and (A) the presence of *RICTOR* amplification, (B) expression of Rictor, and (C) expression of phospho-Akt. Vertical tick marks on curves indicate censored patients.

close to this range. Variations in *RICTOR* amplification among studies most likely reflect inherent differences among various SCLC cohorts. The relatively high frequency of *RICTOR* amplification in our study underlines the importance of this molecular alteration in SCLC.

To our knowledge, this was the first study to use IHC to analyze expression of Rictor and phospho-Akt in SCLC. The sensitivity and specificity of Rictor IHC were 93% and 73%, whereas the sensitivity and specificity of phospho-Akt IHC were 80% and 65%, as compared with *RICTOR* amplification measured by FISH. Only 1 *RICTOR*-amplified tumor showed no Rictor expression in our study. These data suggest that IHC for Rictor may be a useful screening tool to identify patients with SCLC who have a higher likelihood of *RICTOR* amplification.

Some of our cases showed Rictor and phospho-Akt positivity by IHC without *RICTOR* gene amplification by FISH. Possible reasons for this phenomenon include epigenetic modifications or deregulation of miRNA expression, as has been described in several other tumor types [19-23].

Rictor expression at both the mRNA and protein levels has reportedly been higher in distant metastases of adenocarcinoma of lung origin and malignant melanoma compared with that in their primary counterparts [11,24]. Rictor expression was also significantly higher in distant metastases than in primary tumors and lymph node metastases in our study of SCLC. Taken together, these data suggest that Rictor (perhaps as part of mTORC2) has an important role in the development of distant metastases of these tumors.

In a previous study of 42 patients with SCLC, an association was shown between *RICTOR* amplification, as detected by targeted exome sequencing, and decreased survival [4]. The current study could not confirm the same association in a larger cohort of 92 patients using FISH analysis as the diagnostic standard. However, we did observe an association between high expression of Rictor and phospho-Akt and decreased survival, which agrees with observations in several other tumor types [24-29]. These data suggest that high mTORC2 activity, as evidenced by high expression of Rictor and phospho-Akt, may be a better predictor of survival than *RICTOR* amplification.

Rictor is an essential component of the PI3K/AKT/mTOR signaling pathway, one of the most frequently dysregulated pathways in cancer [30]. Previous studies have suggested that *RICTOR* amplification might be a predictive biomarker identifying a subset of patients with lung cancer who have an increased probability of responding to dual mTORC1/2 inhibitor treatment [4,31]. Our study provides further evidence of *RICTOR* amplification as well as new data regarding Rictor and phospho-Akt overexpression in some patients with SCLC. Our data suggest that SCLCs with overexpression of Rictor or phospho-Akt (even without *RICTOR* amplification) may respond to mTORC1/2 therapy. The main limitation of our study is the lack of such clinical information. Hopefully, questions regarding correlation between Rictor and phospho-Akt expression and response to mTORC1/2 therapy will be answered in future clinical trials.

In summary, our results provide a rationale for evaluating dual mTORC1/2 inhibitors as a potential therapy for patients with SCLC, especially for cases in which *RICTOR* amplification is the sole oncogenic driver. IHC staining for Rictor can be used as a highly reliable and cost-effective prescreening method to select cases for *RICTOR* FISH and mTORC1/2 inhibitor therapy.

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