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Original contribution

Expression of mTORC1/2-related proteins in primary and brain metastatic lung adenocarcinoma☆,☆☆,★

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Summary Brain metastases (BMs) are common complications of adenocarcinomas (ADCs) of the lung and are associated with a poor prognosis. Although an increasing amount of data indicates that dysregulated activity of mammalian target of rapamycin (mTOR) can influence the metastatic potential of various tumors, the role of mTOR complexes in the development of BMs from ADCs of the lung is largely unknown. To estimate mTOR activity, we studied the expression of mTOR-related proteins (mTORC1: p-mTOR, p-S6; mTORC2: p-mTOR, Rictor) in primary (n = 67) and brain metastatic (n = 67) lung ADCs, including 15 paired tissue samples, using immunohistochemistry and tissue microarrays. Correlation with clinicopathological parameters was also analyzed. Increased p-mTOR, p-S6, and Rictor expressions were observed in 34%, 33%, and 37% of primary ADCs and in 79%, 70%, and 66% of BMs, respectively. Expression of these markers was significantly higher in BMs as compared with primary carcinomas ($P < .0001$, $P < .0001$, $P < .001$). Rictor expression was significantly higher in primary ADCs of the paired cases with BMs as compared with primary ADCs without BMs (67% versus 28%; $P < .01$). No other statistically significant correlations were found between mTOR activity and clinicopathological parameters. The increased mTORC1/C2 activity in a subset of pulmonary ADCs and the higher incidence of increased mTORC1/

Abbreviations ADC, adenocarcinoma; AKT, protein kinase B; ALK, anaplastic lymphoma kinase; BM, brain metastasis; EGFR, epidermal growth factor receptor; IGFR-1, insulin-like growth factor receptor-1; IHC, immunohistochemistry; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; NSCLC, non–small cell lung cancer; PDGFR, platelet-derived growth factor receptor; PI3K, phosphoinositide 3-kinase; PKC-α, protein kinase C-α; p-mTOR, phosphorylated mTOR; p-S6, phosphorylated ribosomal S6 kinase; Rictor, rapamycin-insensitive companion of mTOR; ROS-1, c-ros oncogene 1; TMA, tissue microarray; VEGFR, vascular endothelial growth factor; mTORI, mTOR inhibitor.

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C2 activity in BMs suggest that the immunohistochemistry panel for characterizing mTOR activity and its potential predictive and prognostic role warrants further investigations.

1. Introduction

Lung cancer, the leading cause of cancer death worldwide, accounts for approximately 40% to 50% of all brain metastases (BMs) [1]. BMs portend a poor prognosis despite the use of multimodal therapies [2,3]. Targetable genetic mutations in non–small cell lung cancer (NSCLC) currently include epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase, and c-ros oncogene 1 [4]. However, to further improve the clinical outcome of patients with brain metastatic NSCLC, additional targets are needed.

The phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway integrates several cell signals and regulates many normal cell functions. It is also one of the most frequently dysregulated signaling pathways in human tumors, including NSCLCs [5]. It can be activated by the EGFR, insulinlike growth factor receptor, vascular endothelial growth factor, and platelet-derived growth factor receptor membrane receptor families and various mutations in the *PI3KCA* gene [6,7]. Activation of the PI3K/AKT/mTOR pathway, in turn, may lead to tumor progression.

The serine/threonine kinase mTOR is an integral part of 2 different multiprotein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Raptor and Rictor are characteristic elements of mTORC1 and mTORC2, respectively. The most important targets of mTORC1 are the S6K and 4EBP-1 proteins. Their phosphorylation results in enhanced protein synthesis through S6 phosphorylation and the release of eukaryotic translation initiation factor 4E. In contrast, mTORC2 regulates the phosphorylation of AKT (Ser473), protein kinase C-α, paxillin and the small GTPases, RAC, and RHO. The 2 complexes differ not only in functions but also in their sensitivity to rapamycin; mTORC1 is sensitive and mTORC2 is considered to be resistant to the drug [8-10]. Long-term treatment with rapamycin, however, can also disrupt mTORC2 assembly and activity [11].

Animal studies have demonstrated that everolimus, temsirolimus, and other next-generation mTOR inhibitors (mTORIs) penetrate the blood-brain barrier [12,13]. In addition, these mTORIs have also shown promise in treating gliomas and breast carcinomas metastatic to the brain [14,15].

To identify potential predictive biomarkers that could guide targeted therapy by mTORIs, we analyzed the expression of mTORC1- and mTORC2-related proteins in primary adenocarcinomas (ADCs) of the lung and brain metastatic ADCs of lung origin.

2. Materials and methods

2.1. Patients and tissue samples

Primary (n = 67) and brain metastatic (n = 67) pulmonary ADCs, including a subset with matched primaries and BMs (n = 15), were studied. The tumors were obtained by surgical resection at the National Korányi Institute of TB and Pulmonology (Budapest, Hungary), the Bajcsy-Zsilinszky Hospital (Budapest), and the National Institute of Clinical Neurosciences (Budapest) between January 2003 and December 2011. The archived tissue samples were used with the approval of the Hungarian Scientific Council National Ethics Committee for Scientific Research (No. 510/2013, 86/2015).

The tumors were re-reviewed and reclassified according to the 2015 *World Health Organization Classification of Lung Tumours* [16]. Clinicopathological data, such as age, sex, stage of primary ADC at diagnosis (as per the 2015 *World Health Organization Classification of Lung Tumours*), smoking status, size, and multiplicity of BMs were obtained from medical records.

Table 1 Summary of antibodies and their conditions used for IHC in this study

Antibody	Clone	Manufacturer	Dilution	Antigen retrieval (buffer)	Detection system
Anti–phospho-mTOR	#2976	Cell Signaling (Danvers, MA)	1:100	Pressure cooker (CA)	Novolink (Novocastra, Wetzlar, Germany)
Anti–phospho-S6	#2211	Cell Signaling	1:100	Pressure cooker (CA)	Novolink
Anti-Rictor	A500-002A	Bethyl Laboratories (Montgomery, TX)	1:1000	Pressure cooker (CA)	Vectastain Elite Universal (Vector Laboratories, Burlingame, CA)

Abbreviation: CA, citric acid buffer (pH 6.0).

Table 2 Clinicopathological characteristics of patients with primary and brain metastatic lung ADCs

		p-mTOR expression			p-S6 expression			Rictor expression		
	Total (%)	"High" (%)	"Low" (%)	*P*	"High" (%)	"Low" (%)	*P*	"High" (%)	"Low" (%)	*P*
Primary ADCs (n = 67)										
Age (mean ± SD), y	61 ± 10	60 ± 11	62 ± 9	.644	62 ± 10	61 ± 10	.540	59 ± 10	62 ± 10	.244
Sex (n = 67)										
Male	25 (37)	5 (20)	20 (80)	.110	8 (32)	17 (68)	1.000	9 (36)	16 (64)	1.000
Female	42 (63)	17 (40)	25 (60)		14 (33)	28 (67)		16 (38)	26 (62)	
Smoking status (n = 51)										
Never smoker	16 (31)	7 (44)	9 (56)	.480	5 (31)	11 (69)	.435	4 (25)	12 (75)	.854
Past smoker	15 (29)	4 (27)	11 (73)		6 (40)	9 (60)		5 (33)	10 (66)	
Current smoker	20 (39)	5 (25)	15 (75)		4 (20)	16 (80)		5 (25)	15 (75)	
Stage of ADC at diagnosis (n = 56)										
I	28 (49)	7 (25)	21 (75)	.147	8 (29)	20 (71)	.120	6 (21)	22 (79)	.067 *
II	7 (13)	2 (29)	5 (71)		0 (0)	7 (100)		1 (14)	6 (86)	
III	12 (21)	5 (42)	7 (58)		4 (33)	8 (67)		4 (33)	8 (67)	
IV	9 (16)	6 (67)	3 (33)		5 (56)	4 (44)		6 (67)	3 (33)	
Brain metastatic ADCs (n = 67)										
Age (mean ± SD), y	60 ± 9	61 ± 10	59 ± 5	.820	61 ± 9	60 ± 8	.696	59 ± 9	63 ± 9	.556
Sex (n = 67)										
Male	37 (55)	27 (73)	10 (27)	1.000	23 (62)	14 (38)	.179	25 (68)	12 (32)	.221
Female	30 (45)	22 (73)	8 (27)		24 (80)	6 (20)		15 (50)	15 (50)	
Size of BM (n = 39)										
<21 mm	19 (49)	13 (68)	6 (32)	.273	16 (84)	3 (16)	.155	9 (47)	10 (53)	.341
≥21 mm	20 (51)	17 (85)	3 (15)		12 (60)	8 (40)		13 (65)	7 (35)	
Multiplicity of BM (n = 49)										
Solitary	37 (76)	26 (70)	11 (30)	.747	24 (65)	13 (35)	.178	26 (70)	11 (30)	.061 *
Multiplex	12 (24)	10 (83)	2 (17)		11 (92)	1 (8)		4 (33)	8 (67)	

* $P \leq .10$ was defined as trend.

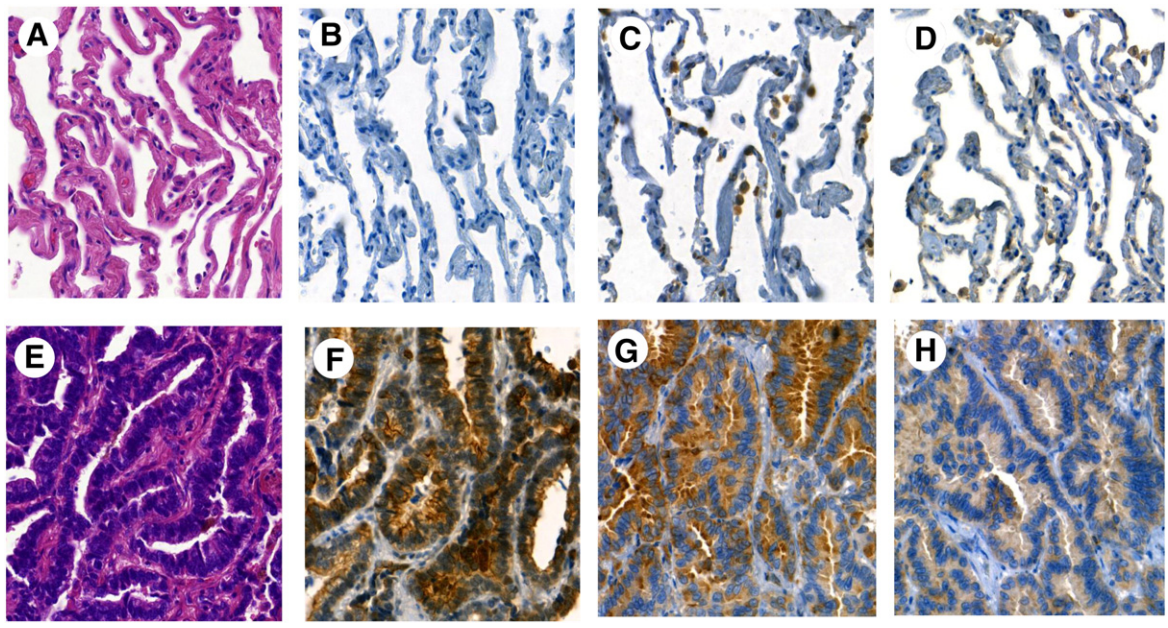

Fig. 1 p-mTOR, p-S6, and Rictor expression in nonneoplastic lung and pulmonary ADC. Hematoxylin and eosin staining (A, nonneoplastic; E, primary ADC); p-mTOR expression (B, nonneoplastic; F, primary ADC); p-S6 expression (C, nonneoplastic; G, primary ADC); and Rictor expression (D, nonneoplastic; H, primary ADC) (original magnification ×400).

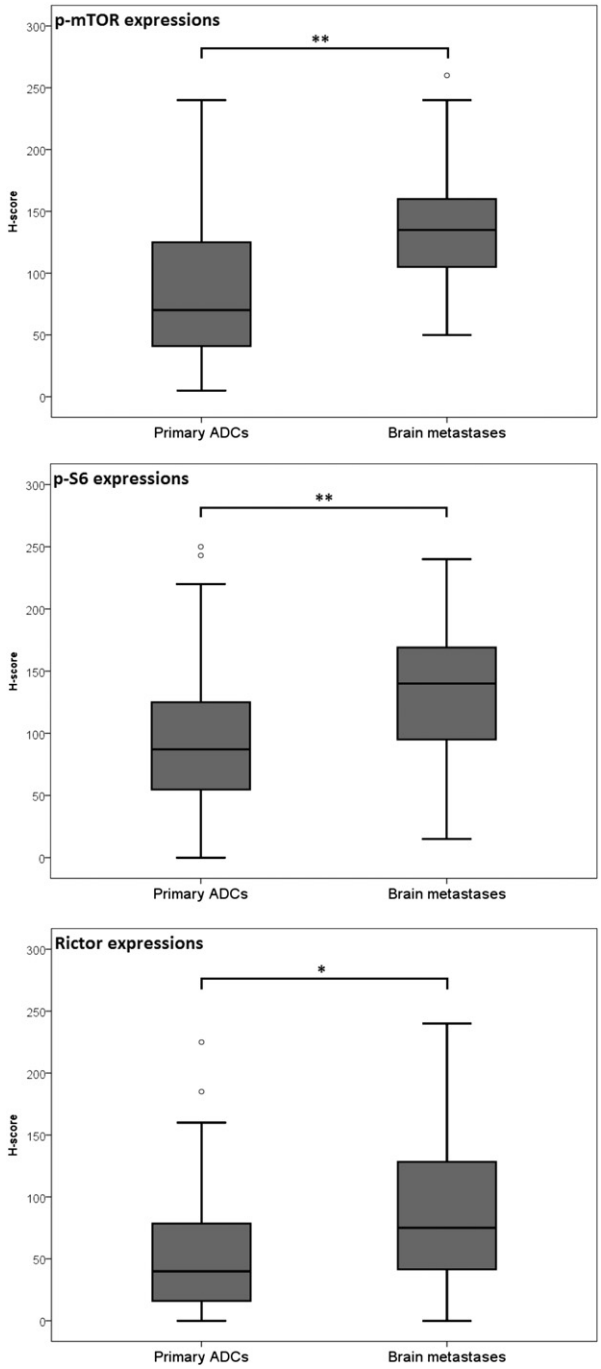

Fig. 2 Evaluated expression levels of p-mTOR, p-S6, and Rictor in primary ADCs and in BMs. Expressions (*H* score) of p-mTOR ($P < .0001$), p-S6 ($P < .0001$), and Rictor ($P < .001$) were significantly higher in BMs than in primary ADCs. *$P < .001$ and **$P < .0001$ calculated with Mann-Whitney *U* test.

2.2. Tissue microarray construction and immunohistochemistry

Tissue microarrays (TMAs) (7 × 10; diameter, 2 mm) with double or triple cores per patient were prepared from selected areas of the formalin-fixed, paraffin-embedded tissue samples (TMA Master; 3DHistech, Budapest, Hungary). Nonneoplastic lung parenchyma, tonsil, placenta, liver, and kidney samples as well as the peritumoral normal tissues were used as controls. The characteristics of the antibodies used in this study are summarized in Table 1.

A scale of 0-300 (*H* score) was used for semiquantitative analysis of immunoreactivity by 2 independent pathologists. The *H* score was calculated by multiplying the fraction of positively stained tumor cells (percentage) by staining intensity (0, 1+, 2+, or 3+), as previously described by Adamo et al [14]. The final *H* score was determined by averaging the *H* scores of all the cores from the same tumor. Cutoff values were defined based on the median score of the tumor samples. Above-median expression was considered "high," whereas expression values below the median were classified as "low."

2.3. Statistical analysis

Mann-Whitney *U* test and Fisher exact test were used for the comparison of the expressions of the mTOR activity markers between primary and brain metastatic lung ADCs and clinicopathological characteristics. All statistical analyses were performed with IBM SPSS Statistics software (version 22; SPSS Inc, Chicago, IL). A $P \leq .05$ (2-tailed) was considered as statistically significant, and $P \leq .10$ was defined as trend.

3. Results

3.1. Clinicopathological characteristics

The expressions of the mTOR signaling–related proteins (p-mTOR, p-S6, Rictor) were studied in primary ADCs (n = 67) and in BMs of ADCs (n = 67), including 15 paired samples. Available clinicopathological information is summarized in Table 2. For patients with paired samples (6 men, 9 women), the mean time interval between the original surgery and the central nervous system recurrence was 25.8 ± 19.3 months.

3.2. Expression of p-mTOR, p-S6, and Rictor in the nonneoplastic lung and primary and brain metastatic lung ADCs

Examples of p-mTOR, p-S6, and Rictor expression in the nonneoplastic lung and primary ADCs of the lung are shown in Fig. 1. Expressions of all 3 markers were low in the nonneoplastic lung. Faint staining was noted in type 1 and type 2 pneumocytes. In tumors, p-mTOR, p-S6, and Rictor proteins were localized mainly in the cytoplasm, but in a few cases, the p-mTOR staining showed nuclear positivity (in 15% of primary ADCs and in 34% of BMs). Rictor appeared in the plasma membrane (<10%) in some cases, but never in the nucleus.

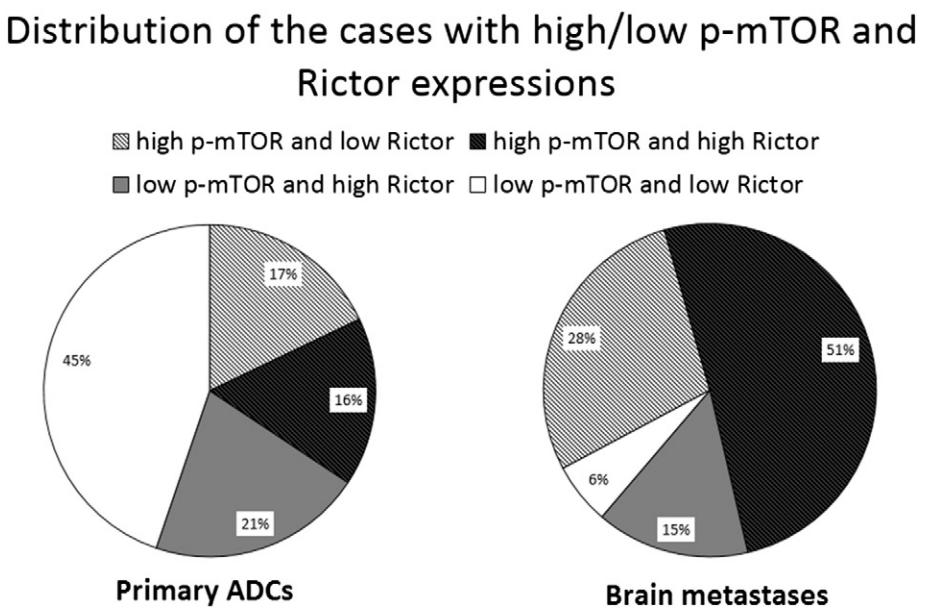

Fig. 3 Distribution of the cases with high/low p-mTOR and Rictor expressions. Expressions of p-mTOR and Rictor in primary and brain metastatic ADCs. Increased mTOR kinase activity (high p-mTOR) or high mTORC2 activity (concordance of high p-mTOR and Rictor expression) was found in 17% and 16%—sum, 33%—of the primary ADCs and in 28% and 51%—sum, 79%—of the BMs, respectively. Striated area indicates increased p-mTOR expression (with or without elevated Rictor expression).

Elevated expressions of p-mTOR (*H* score ≥ 110), p-S6 (*H* score ≥ 115), and Rictor (*H* score ≥ 60) were detected in 33%, 34%, and 37% of the primary ADCs and in 79%, 70%, and 66% of the BMs, respectively. The mean score values for p-mTOR (84 ± 55 versus 136 ± 49; $P < .0001$), p-S6 (97 ± 61 versus 136 ± 54; $P < .0001$), and Rictor (54 ± 48 versus 87 ± 57; $P < .001$) were significantly higher in BMs than in primary ADCs (Fig. 2).

Concordance between p-mTOR and p-S6 status was present in 72% (96/134) of the cases; 58% (22/38) of the discordant cases represented a reduction in p-S6 levels relative to p-mTOR. Moreover, 45% (10/22) of the cases with high p-mTOR and low p-S6 expressions showed increased Rictor immunoreactivity. The expressions of both p-mTOR and Rictor were high in 16% (11/67) of the primaries and in 51% (34/67) of the BMs, whereas high p-mTOR or high Rictor expressions were seen in 18% (12/67) and 21% (14/67) of the primary ADCs and 28% (19/67) and 15% (10/67) of the BMs, respectively. Both p-mTOR and Rictor expressions were low in 45% (30/67) of the primaries and only in 6% (4/67) of the brain metastatic ADCs (Fig. 3).

3.3. Estimation of mTORC1 and mTORC2 activities in primary and brain metastatic lung ADCs

mTORC1 and mTORC2 activities were estimated based on expression of p-mTOR, p-S6, and Rictor (Fig. 4). p-mTOR staining correlates with the active mTOR kinase, which is a component of both complexes. Expression of p-S6 correlates with mTORC1 activity (mTORC1 activates p70S6K, which phosphorylates ribosomal S6), whereas expression of Rictor (an element of mTORC2) correlates with amount of mTORC2.

3.4. Expression of p-mTOR, p-S6, and Rictor in relation to clinicopathological parameters

No statistically significant differences in clinicopathological parameters were found between patients with "high" and "low" p-mTOR, p-S6, and Rictor expressions in either primary or brain metastatic ADCs. However, a statistical trend ($P \leq .10$) between higher stage and increased Rictor expression was observed in primary ADCs ($P = .067$). Moreover, elevated Rictor expressions were seen in a higher number of solitary than multiple metastases (70% versus 33%; $P = .061$) In contrast, there were a higher number of patients with increased p-S6 expression in multiplex than in solitary BMs (65% versus 92%; $P = .178$) (Table 2).

3.5. Expression of p-mTOR, p-S6, and Rictor in paired tumor samples

In the paired tumor samples, p-mTOR, p-S6, and Rictor expressions were altered between the primary and metastatic tumor in 60% (9/15), 60% (9/15), and 40% (6/15) of cases, respectively. The mTORC1 activity increased in most BMs with altered p-mTOR and p-S6 status (p-mTOR: 6/9, p-S6: 8/9); however, the expressions of Rictor were already high in 67% (10/15) of primaries (Fig. 5). High expression of Rictor was seen in 67% of the brain metastatic primaries of the paired cases and 28% of the primary ADCs without BMs ($P < .01$).

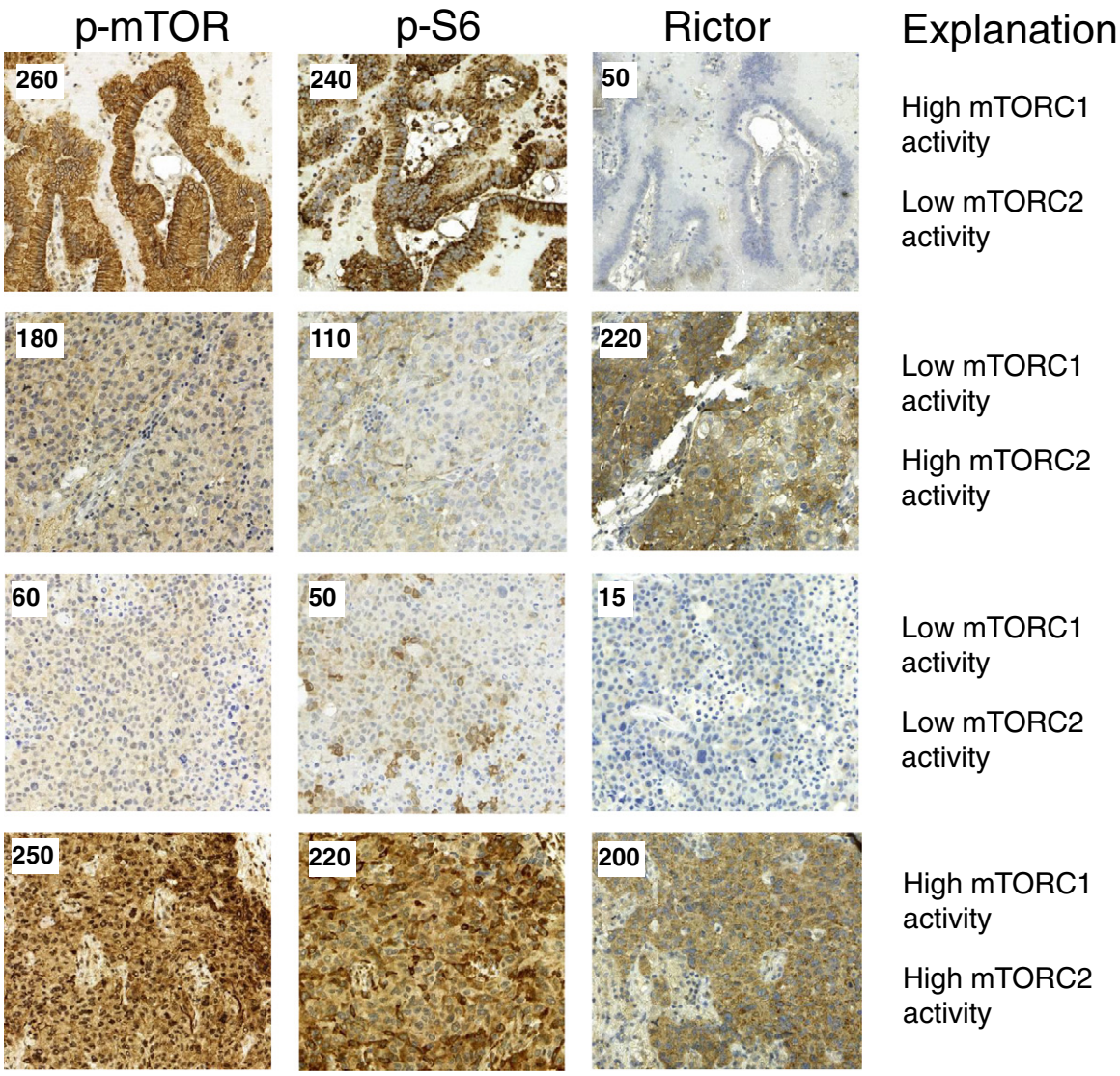

Fig. 4 mTORC1 and mTORC2 activities—immunohistochemical stainings and their evaluation. Different p-S6, p-mTOR, and Rictor score values (in the upper left corners) and the activities of mTORC1 and mTORC2 in BMs of lung ADC cases (original magnification ×200).

4. Discussion

In this study, we used a novel IHC panel to characterize mTORC1 and C2 activities in primary and brain metastatic lung ADCs. The IHC panel consists of p-mTOR, p-S6, and Rictor antibodies. Expression of p-mTOR and p-S6 correlates with mTORC1 activity, whereas expression of p-mTOR and Rictor correlates with mTORC2 activity. Regardless of the clinicopathological characteristics, approximately 30% of the primary and approximately 70% of the brain metastatic ADCs showed increased mTORC1 activity. These results are consistent with previous publications showing hyperactivation of the mTOR pathway in lung cancer [17-20]. Dobashi et al [19] have described a higher frequency of mTOR activation in primary lung ADC cases as compared with normal lung tissue. Liu et al [20] have observed significantly increased mTOR/p-mTOR, P70S6K/p-P70S6K, and p-Akt1 Ser473/Thr308 protein levels in NSCLCs as compared with normal controls. Activation of the mTOR pathway has also been correlated with distant metastases and shorter overall survival in their study [20]. Further studies have shown that the mTOR axis is activated in premetastatic stages of cancer and that expression of mTOR pathway components is enhanced upon metastatic spread [21,22]. In brain metastatic lung cancers, p-mTOR expression has been reported in 31.4% of the tumors [23]. Our results are consistent with these findings and suggest that the mTOR pathway plays a crucial role in metastatic spread of lung ADCs to the brain.

Cheng et al [24] have recently described amplification of *RICTOR* in 13% of more than 1000 lung cancers (in 10.3% of ADCs). Moreover, *RICTOR* amplification has been the sole potentially targetable genomic alteration in 11% of 85 lung cancer cases. Rictor has been found to be a critical mediator

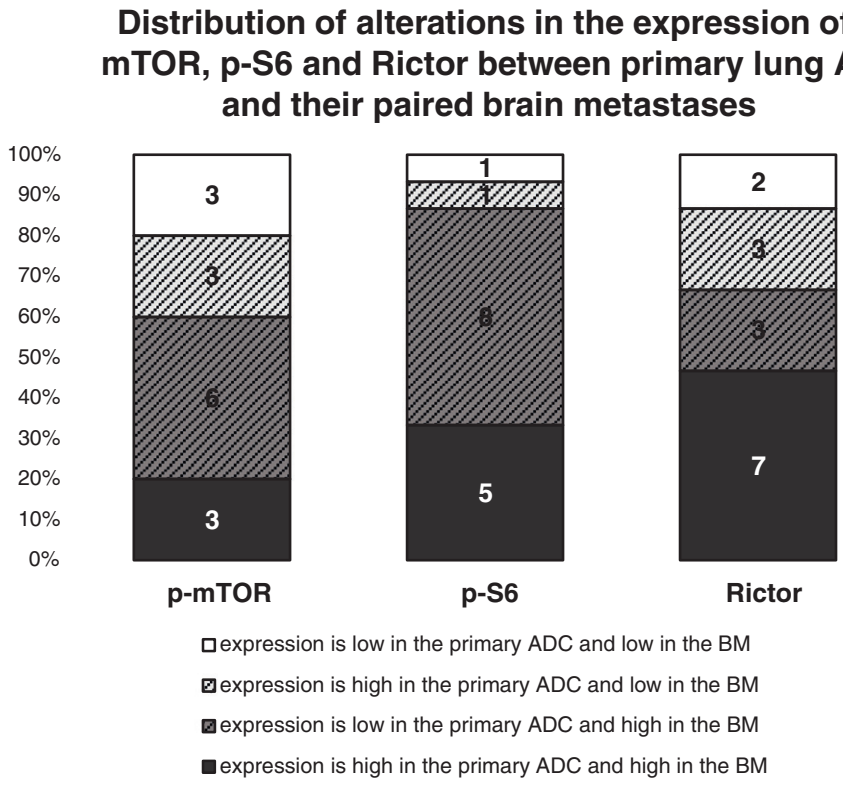

Fig. 5 Distribution of alterations in the expression of p-mTOR, p-S6, and Rictor between primary lung ADCs and their paired BMs. Striated areas indicate cases with alteration of p-mTOR, p-S6, and Rictor expression between primary and metastatic tumors.

of cancer cell metastasis, and Rictor expression is associated with increased lymph node metastases in breast cancers [25]. In our study, increased Rictor expression was detected in 67% (10/15) of primary ADCs of the paired tumor cases (versus 28% of the unpaired cases with no information about metastasis formation). Moreover, increased Rictor expression was associated with higher stage of primary ADCs. These findings suggest that increased Rictor expression may have predictive and prognostic significance in pulmonary ADC.

A recent study has reported coexpression of mTOR, Raptor, and Rictor in 47% of various primary tumors, including 53 of 101 lung cancers that displayed metastases to the brain [22]. Similarly, we found high mTORC1 and C2 activity in 33% of 15 paired primary ADCs that have metastasized to the brain. High mTORC2 activity in 16% of primary ADCs and 51% of BMs underscores the importance of dual mTORC1/2 inhibitors in selected cases.

Despite promising initial results, clinical trials with mTORC1 inhibitors administered singly or in combination with other anticancer agents have shown only modest success in lung cancer patients [26-29]. The insufficient therapeutic response can be related to the nonspecific selection of the patients for mTORI treatment and the activated negative feedback due to mTORC1 inhibition [28,30]. Improved strategies and evaluation of active mTORC1 to active mTORC2 ratio are needed to make the inhibition of the PI3K/AKT/mTOR pathway more effective [30]. The predictive significance of p-S6 and p-mTOR overexpression—a sign of increased mTORC1 activity—for mTORC1 inhibitor therapy efficacy has been described in various tumor types [31-33]; however, it has not been yet extensively studied in NSCLC. The IHC panel described in our study and the resulting mTORC1/2 activity profile may aid in a more accurate selection of lung cancer patients with a greater likelihood to respond to mTOR inhibitor therapy. According to our results, p-mTOR, p-S6, and Rictor proteins warrant further investigation as biomarkers for response to mTORIs in NSCLC.

Taken together, the detected high mTORC1 and C2 activity draws attention to the potential efficacy of mTOR pathway inhibition in both primary and brain metastatic lung ADCs with high mTOR activity. The mTORI treatment could slow down the tumor growth, which could have special benefits for neurological symptoms–related BMs. Previous studies have shown limited clinical benefit with mTORIs and their various combinations in unselected lung cancer patients; however, stratifying patients by mTORC1/2 activity may lead to better patient selection and a more effective targeted therapy.

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