Aberrations in the mTOR pathway and tumor metabolism as potential therapeutic targets in lung neoplasms

Ph.D. Thesis

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1. INTRODUCTION

Lung cancer is the leading cause of cancer death worldwide, with an extremely high incidence and mortality rate in Central and Eastern European countries, including Hungary. In our study, we focused on the mTOR activity and related metabolic alterations in lung adenocarcinomas (ADCs), small cell lung carcinomas (SCLCs), and lymphangioleiomyomatosis (LAM).

As a master regulator of homeostasis, the mTOR pathway integrates several environmental signals to regulate cell growth, proliferation, and metabolism. mTOR is a serine/threonine kinase, which forms the catalytic subunit of two structurally and functionally distinct protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The mTOR complexes differ in their protein components, subcellular localization, rapamycin sensitivity, upstream regulation, and downstream effectors. While mTORC1 primarily regulates fundamental processes that are involved in promotion of cell growth, proliferation, and metabolism, mTORC2 rather controls survival and cell migration by reorganizing the actin cytoskeleton. The mTOR kinase functions as an integral part of the PI3K/Akt/mTOR axis, moreover, interacts with other crucial cascades (e.g., Ras/RafMEK/ERK), establishing a central junction of signaling pathways.

Hyperactivation of mTOR signaling frequently occurs in human neoplasms. According to recent advances in molecular profiling, genetic aberrations of certain genes coding proteins of the mTOR signaling pathway (e.g., *PIK3CA*, *PTEN*, *STK11*, and *RICTOR*) can contribute to mTOR hyperactivation. These genetic alterations are also frequently observed in lung neoplasms and may serve as therapeutic targets.

ADC represents the most common subtype of lung cancers with heterogeneity in its clinical presentation, radiological and histological appearance. ADCs have a high metastatic potential and a poor prognosis, the 5-year survival is only around 15-20%. Aberrant activation of the mTOR pathway has frequently been observed in ADCs. Furthermore, mTOR activation can also be affected by diverse

genetic alterations of many associated signaling pathways, including mutations causing constitutive activation of *EGFR* and *KRAS*.

SCLC accounts for 15-20% of all lung cancers and, because of the rapid tumor growth and early metastatic dissemination, remains a challenging disease with no significant improvement in the therapeutic options in the past decades. Genetic alterations frequently occur in genes that are involved in the PI3K/Akt/mTOR pathway, moreover, *RICTOR* amplification has been identified as the most common targetable gene alteration in SCLC.

LAM is a rare progressive cystic lung disease, which is now considered as a low-grade neoplasm of the perivascular epithelioid cell tumor family. Both sporadic (S-LAM) and tuberous sclerosis-associated LAM (TSC-LAM) are primarily caused by mutations in *TSC1* or *TSC2* genes, which lead to hyperactivation of the mTOR pathway and subsequently result in the proliferation of LAM cells.

Several mTOR pathway inhibitors are under clinical development; but to date, only a few compounds have been approved for the treatment of different tumors. Most inhibitors of the mTOR signaling have limited single-agent activity, however, combination strategies might be useful to increase efficacy. To improve the clinical translation of mTOR pathway inhibitors, it is necessary to identify predictive biomarkers that can facilitate treatment decisions. Besides the biomarker-based patient selection, it would also be important to develop rational drug combinations as well as less toxic and more effective dosing schedules in order to overcome primary and acquired resistance.

2. OBJECTIVES

Despite the several studies that have investigated inhibitors of the PI3K/Akt/mTOR pathway, only a few compounds have been approved for the treatment of cancer. Lack of predictive biomarkers for patient stratification can be a major barrier to the clinical translation of mTOR pathway inhibitors. A better understanding of mTOR signaling may help the translation of next-generation mTOR inhibitors into the clinical setting. Therefore, we aimed to investigate the activity of the mTOR complexes in lung neoplasms and, in relation to study the mTOR pathway, the expression of proteins involved in tumor metabolism in LAM according to the following objectives:

1. Immunohistochemical analysis of mTORC1 and mTORC2 activity and its relations to the clinicopathological data in primary lung ADCs and brain metastatic ADCs of lung origin.

2. Analysis of *RICTOR* amplification and mTORC2 activity in SCLCs:

   - Incidence of *RICTOR* amplification and expression of Rictor and p(Ser473)-Akt in SCLCs in relation to clinicopathological data and survival.

   - The effect of PI3K/Akt/mTOR pathway inhibitors on the proliferation of SCLC cell lines harboring *RICTOR* amplification or other genetic alteration affecting the mTOR signaling *in vitro*.

3. Analysis of mTORC1/2 activity and, in relation to this, the expression of key metabolic enzymes in association with clinicopathological data in LAM.

3. METHODS

Patients

ADC cohort

Primary (N=67) and brain metastatic (N=67) lung ADCs, including a subset with matched primary tumors and brain metastases (N=15), were studied. Tumors were surgically removed at the National Korányi Institute of Pulmonology (Budapest, Hungary), Bajcsy-Zsilinszky Hospital (Budapest, Hungary) and National Institute of Clinical Neurosciences (Budapest, Hungary) (ETT-TUKEB 510/2013 and 86/2015). Tissue microarray blocks were constructed from the archived ADC samples. Available clinical data (age, sex, smoking history, stage at diagnosis, size and multiplicity of the brain metastasis) were also collected.

SCLC cohort

A total of 100 samples (80 cell blocks, 4 transbronchial biopsies, and 16 surgical samples) obtained from 92 patients were analyzed. Six patients had 2 different and 1 patient had 3 different samples from various collection dates. Sample collection was performed at Mayo Clinic (Jacksonville, FL, USA; IRB#: 18-001887). Primary tumors (N=30), lymph node metastases (N=52) and distant metastases (N=18) were also analyzed. Survival data were also available. No patients received chemotherapy before the biopsy.

LAM cohort

We analyzed surgically removed lung tissues of 11 S-LAM patients, who underwent lung transplantation (7 patients) or diagnostic wedge biopsy (4 patients) at Mayo Clinic (Jacksonville, FL, USA; IRB#: 15-000406). Clinicopathological parameters – such as age, smoking history, lymph node metastasis, type of the surgical procedure, treatment before the procedure – were also collected. Two patients

received hormone therapy and none of the patients received mTOR inhibitor prior to surgery.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on 4 μm-thick sections of formalin-fixed paraffin-embedded tissues. After deparaffinization and endogenous peroxidase blocking, antigen retrieval was performed in a pressure cooker for 30 minutes (buffers: citrate pH 6.0; Target Retrieval Solution buffer pH 6.1; 10 mM Tris-EDTA pH 9.0). Slides were incubated with diluted primary antibodies. mTOR kinase activity was characterized by p-mTOR expression. mTORC1 activity was evaluated by expression of the downstream protein p-S6, whereas mTORC2 activity was estimated by expression of p(Ser473)-Akt (p-Akt, downstream target of mTORC2) and Rictor (scaffold protein of mTORC2). Glycolysis and oxidative phosphorylation were characterized by the expression of glucose transporter 1 (GLUT1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-F1-ATPase (ATPB), glutaminolysis by glutaminase (GLS), fatty acid β-oxidation by carnitine palmitoyltransferase 1A (CPT1A), and acetate utilization by acyl-coenzyme A synthetase short-chain family member 2 (ACSS2) and monocarboxylate transporter 1 (MCT1). The expression of LAM markers (SMA, HMB-45 and β-catenin) and hormone receptors (estrogen and progesterone) was also examined in LAM samples. Immunohistochemical reactions were visualized by Novolink Polymer (Leica) and Vectastain Universal Elite ABC HRP Kit (Vector) detection systems. 3,3'-diaminobenzidine (DAB) was used as chromogen, and sections were counterstained with hematoxylin.

Immunostained sections were reviewed independently by 2 investigators. For mTOR and metabolic markers, the H-score was calculated in the ADC and LAM samples. In SCLCs, the percentage of positive cells of any intensity was determined. The expression of the markers was categorized as follows: low/high expression (ADCs),

no/low/moderate/high expression (SCLCs), no/low/high expression (LAM).

RICTOR fluorescence *in situ* hybridization

Sections of formalin-fixed, paraffin-embedded cell blocks and tissue blocks were used for fluorescence *in situ* hybridization (FISH) analysis. After deparaffinization and pretreatment (79°C, 25 min), protease digestion was performed at 38°C for 25 minutes. Probes for *RICTOR* (#RICTOR-20-OR; Empire Genomics) and chromosome 5 (*Chr5*) control (#CHR05-10-GR; Empire Genomics) were mixed with hybridization buffer and hybridized at 37°C for 2 hours. After a posthybridization wash (73°C, 3 min) and air drying, sections were counterstained with DAPI I.

Representative areas were selected and examined under a fluorescence microscope using CytoVision software (Leica). Focusing on hot spots, orange *RICTOR* and green *Chr5* signals were counted by 2 investigators in 30 tumor cell nuclei from at least 2 different areas. For each tumor, the mean number of signals per nucleus was determined, and the *RICTOR/Chr5* ratio was established. The samples were categorized as positive, negative, and equivocal.

In vitro experiments

Cell lines

Human SCLC cell lines harboring *RICTOR* amplification or other genetic alteration affecting the mTOR signaling were used in our study: H196 (*RICTOR* amplification, *PTEN* mutation), H1048 (two missense mutation in the *PIK3CA* gene, *AKT3* amplification), H146 (*PIK3CA* and *AKT1* amplification), and DMS153 (*PIK3CA* amplification and *RICTOR* missense mutation).

Alamar Blue assay

Alamar Blue assay was used to assess the effect of mTOR pathway inhibitors on the proliferation of the SCLC cells after 72 h treatment. Alamar Blue (Thermo Fisher Scientific) was added to the plates at a final concentration of 10% and the fluorescent color change was monitored after 4 hours using Fluoroskan Ascent FL fluorimeter. The relative cell proliferation was calculated as the percentage of untreated cells. The following inhibitors were used: cisplatin (3 μM, standard chemotherapy), rapamycin (50 ng/mL, mTORC1 inhibitor), PP242 (1 μM, mTORC1/2 inhibitor), vistusertib (1 μM, mTORC1/2 inhibitor), dactolisib (1 μM, PI3K and mTORC1/2 inhibitor), and ipatasertib (1 μM, Akt inhibitor).

Statistical analysis

For parametric data, two-sample t-test was used when comparing two groups, whereas non-parametric data were analyzed with Mann-Whitney *U* test and Wilcoxon signed-rank test. Fisher's exact test was performed to compare categorical variables. Correlations were assessed using Spearman's rank correlation. The Kaplan-Meier method was performed to estimate survival, and the difference between survival curves was compared by the log-rank test. Statistical significance was defined as $P \leq .05$ for ADC and SCLC samples and, in order to achieve highly reliable results, $P \leq .01$ for LAM samples.

4. RESULTS

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

The expressions of the mTOR signaling-related proteins (p-mTOR, p-S6, Rictor) were studied in primary lung ADCs (N=67) and brain metastases (BMs) of lung ADCs (N=67), including 15 pairs of matched primary tumors and metastases. Activity of mTORC1 and mTORC2 was estimated based on expression of p-mTOR, p-S6, and Rictor.

Expression of p-mTOR, p-S6, and Rictor were low in the peritumoral normal lung tissue, faint staining was observed both in type 1 and type 2 pneumocytes. In tumor cells, p-mTOR, p-S6, and Rictor proteins were localized mainly in the cytoplasm, however, in a few cases, p-mTOR staining also showed nuclear positivity (in 15% of primary ADCs and in 34% of BMs). Rictor staining was also observed in the plasma membrane in less than 10% of the cases, but never in the nucleus.

High expression of p-mTOR, p-S6, and Rictor 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 all 3 markers were significantly higher in BMs than in primary ADCs ($P < .01$). No statistically significant association was observed with the clinicopathological parameters.

Concordance between p-mTOR and p-S6 expression was present in 72% (96/134) of the cases; 58% (22/38) of the discordant cases represented a decrease in p-S6 expression relative to p-mTOR. Moreover, 45% (10/22) of the cases with high p-mTOR and low p-S6 levels showed increased Rictor immunoreactivity. High expression of both p-mTOR and Rictor were observed in 16% (11/67) of the primary ADCs and in 51% (34/67) of the BMs.

In the paired samples, p-mTOR, p-S6, and Rictor expressions altered between the primary ADCs and BMs in 60% (9/15) and 40% (6/15) of the 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). Elevated expression of Rictor was detected in 67% (10/15) of the

brain metastatic primary ADCs as compared to 28% of the primary ADCs without BMs ($P < .01$).

Analysis of *RICTOR* amplification and mTORC2 activity in small cell lung cancer

RICTOR amplification was analyzed using FISH in 100 SCLC samples. 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 (15%), whereas 3 cases (3%) were equivocal and 82 cases (82%) were negative for *RICTOR* amplification.

Expression of Rictor and p-Akt was analyzed by IHC in the SCLC samples. The expression of Rictor was high in 14 cases (14%), moderate in 23 cases (23%), low in 25 cases (25%), and we observed no Rictor expression in 38 cases (38%). The expression of p-Akt was high in 16 cases (16%), moderate in 26 cases (26%), low in 35 cases (35%), and we observed no p-Akt expression in 23 cases (23%). For sensitivity and specificity analysis, high and moderate expression was categorized as 'high', whereas low and no expression was categorized as 'low'.

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

Of the 15 *RICTOR*-amplified cases, 14 (93%) were positive for Rictor (5 high and 9 moderate expression), and 12 (80%) were positive for p-Akt (5 high and 7 moderate expression). Only one case with *RICTOR* amplification was negative for both Rictor and p-Akt. In contrast, among the 85 cases with no *RICTOR* amplification (negative or equivocal), 23 (27%) were positive for Rictor (9 high and 14 moderate expression), and 30 (35%) were positive for p-Akt (11 high and 19 moderate expression). Considering *RICTOR* FISH as a gold standard method, the sensitivity and specificity were 93% and 73% for Rictor IHC and 80% and 65% for p-Akt IHC, respectively.

We found no significant associations between the presence or absence of *RICTOR* gene amplification and clinicopathological parameters, however, both Rictor ($P < .001$) and p-Akt ($P = .09$) expression was higher in distant metastases than in lymph node metastases or primary tumors. There was no association between the presence of *RICTOR* amplification and overall survival. In contrast, high expression of either Rictor ($P = .007$) or phospho-Akt ($P < .001$) was associated with significantly decreased overall survival compared to cases with no expression of the same marker.

In our *in vitro* studies, we analyzed the effect of mTOR pathway inhibitors on the proliferation of SCLC cell lines harboring *RICTOR* amplification or other genetic alterations affecting the PI3K/Akt/mTOR pathway.

Using cell blocks of the SCLC cell lines, we proved the presence of *RICTOR* amplification in the H196 cell line, whereas H1048, H146 and DMS153 cell lines showed no copy number variation in the *RICTOR* gene.

The cell lines showed different cisplatin sensitivity. The *RICTOR* amplified H196 cells were resistant, whereas cisplatin resulted in a ~50% ($P<.05$) decrease in the proliferation of DMS153 cell line harboring a missense mutation in the *RICTOR* gene.

Rapamycin, an mTORC1 inhibitor, had a slight antiproliferative effect on H196 cells and resulted in a 40-50% decrease in the proliferation of the H1048 and H146 cell lines. *RICTOR* mutant DMS153 cells were, however, resistant to the rapamycin treatment.

The effects of mTORC1/2 inhibitors PP242 and vistusertib were also studied. PP242 resulted a remarkable antiproliferative effect on the proliferation of H196, H1048 and H146 cell lines, however, it resulted only a 20% decrease in the proliferation of the rapamycin resistant DMS153 cells. Vistusertib had a significant (>40%) antiproliferative effect in all studied cell lines.

The antiproliferative effects of the PI3K/mTOR inhibitor dactolisib were similar to rapamycin, whereas ipatasertib, a pan-Akt inhibitor, did not have any significant effect on the proliferation of the SCLC cell lines tested.

Expression of mTORC1/2 and cellular metabolism-related proteins in lymphangioleiomyomatosis

The expression of mTORC1/2 (p-S6 and Rictor) and cellular metabolism-related (GLUT1, GAPDH, ATPB, GLS, ACSS2, MCT1, and CPT1A) markers were studied in 11 S-LAM cases. In absence of proven precursor for LAM cells, we used bronchial smooth muscle (BSM) cells as controls.

In LAM cells, high expression of p-S6 suggesting high mTORC1 activity was detected in 10 of 11 cases (91%), whereas high expression of Rictor suggesting high mTORC2 activity was seen in 6 of 11 cases (55%). Low expression of both p-S6 and Rictor was detected in only 1 case. In contrast, no or low p-S6 and Rictor expression was observed in BSM cells in all cases. The difference in p-S6 and Rictor expression was significant between LAM and BSM cells ($P < .01$).

GLUT1 positivity was observed in LAM cells in 5 of 11 cases (45%), the expression was high in only 1 case. Minimal or no GLUT1 expression was detected in BSM cells in all cases. We found no significant difference in GLUT1 H-scores between LAM and BSM cells ($P = .61$).

Cytoplasmic immunoreactivity was evaluated for GAPDH and ATPB. In LAM cells, high expression of GAPDH and ATPB was detected in 7 (64%) and 4 (36%) cases, respectively. In contrast, we observed high expression of ATPB in only 1 case (9%) and high expression of GAPDH in none. The difference in H-scores between LAM and BSM cells was not statistically significant for ATPB ($P = .02$), however, GAPDH expression was significantly higher in LAM cells ($P < .01$).

LAM cells showed GLS expression in 10 of 11 cases (91%); the expression was high in 7 cases (64%). On the other hand, GLS expression was absent in 91% of the cases in BSM cells. The H-score for GLS was significantly higher in LAM cells compared to BSM cells ($P < .01$).

Expression of CPT1A was high in both LAM and BSM cells in all cases. Nevertheless, the mean H-score for CPT1A was significantly higher in LAM cells compared to BSM cells ($P < .01$).

While MCT1 and ACSS2 expressions were low or absent in BSM cells, we observed high MCT1 and ACSS2 expression in 7 (64%) and 9 (82%) cases, respectively. The H-scores for MCT1 and ACSS2 were significantly higher in LAM cells than in BSM cells ($P < .01$).

Correlations between the expression of p-S6, Rictor and metabolic pathway-related proteins and transporters were also analyzed. In LAM cells, positive correlation was observed between p-S6 and GLS ($\rho = 0.732$, $P = .01$) and between Rictor and ACSS2 ($\rho = 0.849$, $P < .01$). In addition, a positive correlation was also detected between Rictor and ACSS2 in BSM cells ($\rho = 0.769$, $P < .01$).

The expression of both Rictor and ATPB was higher in explanted (end-stage) lungs than in diagnostic wedge biopsies. A significant positive correlation was also detected between CPT1A and estrogen receptor positivity in LAM cells ($\rho = 0.808$, $P < .01$). No other association was observed between protein expression and clinicopathological data.

5. CONCLUSIONS

I. Immunohistochemical analysis of mTORC1/2-related proteins in primary and brain metastatic lung ADCs:

1. High expression of p-mTOR, p-S6 and Rictor was observed in 30-40% of primary ADCs and 65-80% of BMs. The expression of all three markers was higher in BMs than in primary ADCs.

2. The mTOR activity increases during the metastatic process, moreover, high Rictor expression in the primary tumor can predict subsequent metastasis formation.

II. Analysis of *RICTOR* amplification and mTORC2 activity in SCLC:

1. Rictor and p-Akt expression is higher in *RICTOR* amplified cases; Rictor IHC can be used as a prescreening method for *RICTOR* amplification with a high sensitivity (93%) and a moderate-high specificity (73%).

2. The expression of Rictor is significantly higher in distant metastases of SCLCs than in the primary tumors suggesting the role of mTORC2 in the metastatic process.

3. High expression of either Rictor or p-Akt – even without *RICTOR* amplification – is associated with decreased overall survival.

4. mTORC1/2 inhibitors have a strong antiproliferative potential *in vitro* in SCLC cell lines harboring *RICTOR* amplification or other genetic alterations affecting the PI3K/Akt/mTOR pathway.

III. Analysis of mTOR signaling and cellular metabolism-related proteins in LAM:

1. In addition to the increased mTORC1 activity, high Rictor expression in more than half of the cases, and higher Rictor expression in the end-stage lungs indicate the importance of mTORC2 activity in the pathobiology and progression of LAM.

2. Glutaminolysis, fatty acid β-oxidation, and acetate utilization may have an important role in LAM cell metabolism; and the positive correlation between p-S6 and GLS as well as Rictor and ACSS2 suggest that mTOR inhibitors can also influence bioenergetic processes, which may contribute to their therapeutic effects.

6. PUBLICATIONS

Publications related to the thesis:

1.) **Krencz I**, Sebestyén A, Fábián K, Márk Á, Moldvay J, Khoor A, Kopper L, Pápay J. (2017) Expression of mTORC1/2-related proteins in primary and brain metastatic lung adenocarcinoma. Hum Pathol, 62:66-73. IF: 3.125

2.) **Krencz I** *, Sebestyen A *, Papay J, Jeney A, Hujber Z, Burger CD, Keller CA, Khoor A. (2018) In situ analysis of mTORC1/2 and cellular metabolism related proteins in human lymphangioleiomyomatosis. Hum Pathol, 79:199-207. (* contributed equally) IF: 2.740

3.) **Krencz I**, Sebestyen A, Papay J, Lou Y, Lutz GF, Majewicz TL, Khoor A. (2019) Correlation between immunohistochemistry and RICTOR fluorescence in situ hybridization amplification in small cell lung carcinoma. Hum Pathol, 93:74-80. IF: 2.740

4.) **Krencz I**, Sebestyen A, Khoor A. (2020) mTOR in lung neoplasms. Pathol Oncol Res, doi: 10.1007/s12253-020-00796-1. [Epub ahead of print] IF: 2.433

Publications not related to the thesis:

1.) Hujber Z, Petővári G, Szoboszlai N, Dankó T, Nagy N, Kriston C, **Krencz I**, Paku S, Ozohanics O, Drahos L, Jeney A, Sebestyén A. (2017) Rapamycin (mTORC1 inhibitor) reduces the production of lactate and 2- hydroxyglutarate oncometabolites in IDH1 mutant fibrosarcoma cells. J Exp Clin Cancer Res, 36(1):74. IF: 6.217

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4.) Petővári G, Dankó T, **Krencz I**, Hujber Z, Rajnai H, Vetlényi E, Raffay R, Pápay J, Jeney A, Sebestyén A. (2019) Inhibition of metabolic shift can decrease therapy resistance in human high-grade glioma cells. Pathol Oncol Res, doi: 10.1007/s12253-019-00677-2. [Epub ahead of print] IF: 2.433

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