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**Mammalian target of rapamycin signaling activation patterns in neuroendocrine tumors of the lung.**

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# UNIVERSITÀ DEGLI STUDI DI TORINO

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1           **MAMMALIAN TARGET OF RAPAMYCIN (mTOR) SIGNALLING ACTIVATION**  
2           **PATTERNS IN NEUROENDOCRINE TUMORS OF THE LUNG**

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4           **Luisella Righi<sup>1</sup>, Marco Volante<sup>1</sup>, Ida Rapa<sup>1</sup>, Veronica Tavaglione<sup>1</sup>, Frediano Inzani<sup>2</sup>,**  
5           **Giuseppe Pelosi<sup>3</sup>, Mauro Papotti<sup>1</sup>.**

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7           <sup>1</sup>Divisions of Pathology, Department of Clinical & Biological Sciences, University of Turin at San  
8           Luigi Hospital, Orbassano, Torino; <sup>2</sup>Division of Pathology, University of Parma, Parma;  
9           <sup>3</sup>Diagnostic Histopathology Unit, European Institute of Oncology and Department of Medicine,  
10           Surgery and Dentistry at the University of Milan, Milano, Italy.

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12           **Short Title:** mTOR signalling pathway in lung neuroendocrine tumors.

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14           **Keywords:** lung, neuroendocrine tumors, carcinoid, mTOR, signalling.

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18           **Address for correspondence:**

19           Luisella Righi, MD

20           Department of Clinical & Biological Sciences, University of Turin at San Luigi Hospital,

21           Regione Gonzole 10, 10043 Orbassano, Torino, Italy.

22           Tel: +390119026018

23           Fax +390119026753;

24           e-mail: [luisella.righi@unito.it](mailto:luisella.righi@unito.it)

25

1 **ABSTRACT**

2

3 Among alternative therapeutic strategies in clinically aggressive neuroendocrine tumors (NET) of  
4 the lung, promising results have been obtained in experimental clinical trials with mTOR inhibitors,  
5 though in the absence of a proven mTOR signalling activation status. This study was aimed at  
6 analyzing the expression of phosphorylated mTOR (p-mTOR) and of its major downstream  
7 activation molecules p70-S6K (p-S6K) and 4EBP1 (p-4EBP1) in a large series of 218 surgically  
8 resected, malignant lung NETs, including 24 metastatising typical carcinoids, 73 atypical  
9 carcinoids, 60 large cell neuroendocrine carcinomas (LCNEC) and 61 small cell carcinomas  
10 (SCLC). By immunohistochemistry, higher levels of p-mTOR and p-S6K were detected in low-to  
11 intermediate grade tumors in comparison with high grade tumors ( $p < 0.001$ ), at variance with p-  
12 4EBP1 which was mainly expressed in LCNEC and SCLC ( $p < 0.001$ ). Western blot analysis of  
13 NET tumor samples and lung NET cell lines confirmed such findings. A strong correlation between  
14 p-mTOR and p-S6K expression ( $p < 0.0001$ ) proved the activated status of mTOR pathway.  
15 Moreover, p-mTOR protein expression was positively associated with somatostatin receptor(s)  
16 expression. None of the investigated molecules impacted on survival. However, in low grade  
17 tumors, low p-mTOR expression correlated with lymph node metastases ( $p = 0.016$ ), recurrent  
18 disease and survival ( $p = 0.005$ ). In conclusion, these data demonstrate a differential mTOR  
19 activation status in the spectrum of pulmonary NETs, possibly suggesting that mTOR pathway  
20 profiling might play a predictive role in patients candidate for mTOR-targeted therapies.

21

# 1 INTRODUCTION

2

3 The management of lung neuroendocrine tumors (NET) mainly depends on both grade of  
4 differentiation (low-to intermediate *vs* high grade) and clinical stage at diagnosis (localized *vs*  
5 metastatic). Surgery is the treatment of choice for low-to intermediate grade (i.e. typical or atypical  
6 carcinoids) and localized tumors, while in high grade and/or disseminated lesions chemotherapy is  
7 generally preferred (Pelosi *et al.* 2006; Garcia-Yuste *et al.* 2008). Traditional therapies offer limited  
8 benefits to patients with advanced disease: the traditional DNA-damaging cytotoxic agents (i.e.  
9 platinum-based drugs) have low efficacy and although a large number of therapeutic options have  
10 been explored, there is little consensus on a single standard treatment approach (Srirajaskanthan *et*  
11 *al.* 2008), especially in the group of clinically aggressive bronchial carcinoids.

12 Emerging data on the molecular mechanisms of carcinogenesis and tumor progression prompted  
13 a new era of molecular therapeutics with the development of selective targeted agents. In this  
14 context, there are several, yet poorly explored, potential therapeutic options for lung NET including  
15 somatostatin analogs, inhibitors of the VEGF pathway, and inhibitors of the mammalian Target Of  
16 Rapamycin (mTOR) which have shown promising activity in recent clinical studies (Duran *et al.*  
17 2007; Kulke 2007; Yao *et al.* 2008).

18 mTOR is a serine threonine kinase that participates in the regulation of proliferation, cell growth  
19 and apoptosis through modulation of cell cycle progression (Vignot *et al.* 2005). The activated  
20 (phosphorylated) mTOR kinase leads to the subsequent phosphorylation of downstream effectors:  
21 the ribosomal p70S6-kinase (S6K) and the eukaryotic initiation factor 4E-binding protein 1  
22 (4EBP1), two key proteins that regulate translation of mRNAs into proteins required for cell cycle  
23 progression from G1 to S phase (Podsypanina *et al.* 2001; Dancey 2006). Recent insights revealed a  
24 significant complexity of the mTOR pathway that seems to cross talk with other well-characterized  
25 signalling cascades, thus paving the way to the use of combined therapies (Bjornsti and Houghton  
26 2004; Guertin and Sabatini 2007; Meric-Bernstam and Gonzalez-Angulo 2009). mTOR signalling

1 pathway can be upstream activated - most commonly via the PI3 Kinase/AKT pathway - by  
2 receptors such as somatostatin receptors or insulin-like growth factor receptor 1 or by loss of  
3 inhibiting molecules, such as PTEN (von Wichert *et al.* 2000; Wang *et al.* 2002). Rapamycin  
4 (Sirolimus, Wyeth, Philadelphia, PA) and its derivatives are immunosuppressive macrolides that  
5 specifically block mTOR signalling and have been shown to possess anti-proliferative activity in a  
6 variety of malignancies both *in vitro* (Zitzmann *et al.* 2007) and in phase II clinical trials (Yao *et al.*  
7 2008). Inhibition of mTOR prevents phosphorylation of S6K, 4EBP1 and, indirectly, other proteins  
8 involved in the transcription and cell cycle control, leading to G1 phase cell growth arrest.

9 Two rapamycin derivatives have recently been evaluated in patients with NETs: Temsirolimus  
10 (CCI-779; Wyeth, Madison, NJ) and Everolimus (RAD001; Novartis, Basel, Switzerland). A  
11 multicentric study has recently demonstrated that Temsirolimus effectively down-regulates the  
12 phosphorylation of S6K and that higher baseline levels of phospho-S6K and phospho-mTOR seem  
13 to predict a better response in advanced neuroendocrine (NE) carcinomas (Duran *et al.* 2006),  
14 although Temsirolimus does not modified the progression-free survival in advanced small cell lung  
15 cancer patients (Pandya *et al.* 2007). New perspectives flow from phase I trials aimed at  
16 determining the safety, tolerability, pharmacokinetics and pharmacodynamics of novel mTOR  
17 inhibitors such as Deforolimus (AP23573, Ariad Pharmaceuticals, Cambridge, MA), that was well  
18 tolerated and showed encouraging antitumor activity (Mita *et al.* 2008). Furthermore, *in vitro*  
19 studies and *in vivo* clinical trials combining mTOR inhibitors and the somatostatin analog octreotide  
20 have recently been published with controversial results in terms of additive anti-tumoral effects of  
21 the two compounds (Grozinsky-Glasberg *et al.* 2008; Moreno *et al.* 2008; Yao *et al.* 2008).

22 Despite all the above pre-clinical and clinical studies on the anti-neoplastic efficacy of mTOR  
23 inhibitors in a variety of tumors, data on the activation status of mTOR signalling cascade in  
24 pulmonary NET are still lacking. In this respect, a detailed protein expression map of mTOR  
25 pathway-related molecules in lung NET, could not only define specific expression patterns  
26 predictive of clinical response, as suggested for other malignancies (Lam *et al.* 2007), but also

1 investigate the prognostic implications of these molecules. Therefore, aim of this study was to  
2 evaluate the expression of activated mTOR related proteins in a large series of pulmonary NET -  
3 with special reference to clinically malignant cases.

4

## 5 **MATERIALS AND METHODS**

6

7 **Case selection.** Eight hundred eighty three surgically resected NETs of the lung were recorded  
8 between 1989 and 2007 in the pathology files of the Universities of Turin and Parma and the  
9 European Institute of Oncology of Milan (467, 188 and 217 cases, respectively). Among them, a  
10 series of 218 clinically malignant lesions (129 cases from Turin, 40 from Parma and 49 from Milan)  
11 was collected, including 24 typical carcinoids (TC) with lymph node metastases at the time of the  
12 diagnosis (TC mets), 73 atypical carcinoids (AC) with or without lymph node metastases, 60 large  
13 cell neuroendocrine carcinomas (LCNEC) and 61 small cell carcinomas (SCLC). Pathological  
14 samples corresponded to primaries in all but 15 cases, where lymph node metastases were the only  
15 available material. In 15 cases, primary tumor and corresponding lymph node metastasis was  
16 analyzed. All cases were classified according to the last 2004 WHO Classification on Lung Tumors  
17 (Travis *et al.* 2004) and the clinical and pathological characteristics collected elsewhere (Righi *et al.*  
18 2009) and including sex, age, primary tumour size, Ki-67 labeling index (expressed as the  
19 percentage of positive cells in highest labeling areas), nodal status, stage, follow up and site of  
20 distant metastases were detailed in Table 1. Forty consecutive non metastatic TC were raised from  
21 the files of the University of Turin to be used as control group for baseline expression of the  
22 markers under evaluation. All cases were anonymized by a pathology staff member not involved in  
23 the study. Clinical data were compared and analysed through coded data, only. The study was  
24 approved by the institutional review board of the Hospital

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2       **Immunohistochemistry.** Immunohistochemistry was performed using monoclonal antibodies  
3 against Ser2448-phospho-mTOR (p-mTOR, rabbit 49F9, diluted 1/100), Thr389-phospho-p70S6K,  
4 (p-S6K, mouse 1A5, diluted 1/400) and Thr37/46-phospho-4EBP1 (p-4EBP1, rabbit 236B4, diluted  
5 1/300); all antibodies were purchased from Cell Signalling Technologies, Beverly, MA. Five  
6 micron-thick paraffin sections were collected onto charged slides, deparaffinized and re-hydrated in  
7 water. After antigen retrieval in pH 6.0 citrate buffer for 5 minutes at 125°C in a pressure cooker,  
8 the relevant primary antibodies were incubated overnight at 4°C. Immunoreactions were revealed  
9 by a biotin-free dextran-chain detection system (Envision, DakoCytomation, Glostrup, Denmark),  
10 and developed using 3'-3'-diaminobenzidine as the chromogen. The specificity of all reactions was  
11 validated in parallel control sections omitting the primary antibodies for each immunohistochemical  
12 run.

13

14       **Immunohistochemical data interpretation.** Immunohistochemical findings were evaluated  
15 independently by two of us (LR and MV) and cases with conflicting scores were reviewed jointly at  
16 a multi-head microscope until a consensus was reached. All cases were evaluated using a semi-  
17 quantitative histological score (H-score) (Huang *et al.* 2005; Cappia *et al.* 2008) taking into account  
18 both the percentage of positive tumor population within the whole section and the immunostaining  
19 intensity evaluated subjectively as being negative (0), weak (1), moderate (2) and strong (3). For  
20 each case, the H-score was obtained multiplying the percentages of reactive cells by the  
21 corresponding immunostaining intensity obtaining a final score ranging from 0 to 300.

22

23       **Western Blot analysis.** Four lung neuroendocrine tumor cell lines (from typical - H727 - and  
24 atypical - H720 - carcinoids, and from small cell carcinomas, H69 and H526) were available from  
25 ATCC (Manassan, VA, USA). In addition, 14 frozen lung NET samples, not included in the present



1 series of 218 cases, were available from the tissue bank of the Pathology Unit at the University of  
2 Turin.

3 All samples were homogenized and lysated in TNE lysis buffer supplemented with 1% protease  
4 inhibitor cocktail (Complete, Roche Diagnostic Corporation, IN). The protein concentration was  
5 evaluated using BCA protein assay Kit (Pierce, Milwaukee, WI), and 50 micrograms of protein  
6 were resolved in 8% SDS-PAGE and transferred to nitrocellulose membranes for each experiment.  
7 The membrane blots were blocked for 1h with 5% BSA in TBS-Tween 0,1% and incubated  
8 overnight 4°C with primary antibody: anti-p-AKT (Thr 308, 1:1000), anti-p-mTOR ( Ser2884,  
9 1:1000), anti-p-p70S6K (Thr 389, 1: 2000) (all from Cell Signalling Technology, Beverly, MA).  
10 Anti-  $\beta$ -Actin (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) was used as the loading control.  
11 Immunoreactive proteins were visualized using horseradish peroxidase–conjugated anti-mouse or  
12 anti-rabbit antibody (1:3,000 and 1:1,000, respectively) and Enhanced Chemiluminescence (ECL)  
13 (Amersham, Biosciences, Piscataway, NJ) as the substrate.

14  
15 ***Statistical analysis.*** Statistical analysis was performed using Graphpad 4 software and the  
16 results were considered statistically significant at a level of  $p < 0.05$ . One-way ANOVA and non  
17 parametric Mann-Whitney *U* tests were used to compare the distribution of the markers investigated  
18 among the different tumor groups and with respect to clinical pathological variables. The Spearman  
19 test was used to analyze the correlation index among markers expression. Overall survival analysis  
20 was performed using the Kaplan-Meier method and Log-Rank test.

## 21 22 **RESULTS**

23  
24 ***Distribution of mTOR signalling molecules in lung NETs.*** Immunohistochemical staining for  
25 p-mTOR and p-4EBP1 provided the expected cytoplasmic pattern, while p-S6K showed either a  
26 cytoplasmic perinuclear dot-like or a diffuse nuclear pattern of staining.

1 In peri-tumoral non neoplastic parenchyma, a weak p-mTOR, p-S6K and p-4EBP1  
2 immunoreactivity was observed in normal bronchial epithelium and endothelia. Alveolar histiocytes  
3 were also reactive for p-S6K and p-4EBP1, whereas a strong p-mTOR immunoreactivity was  
4 detected in reactive alveolar epithelial cells at the periphery of the tumors (data not shown).

5 Distribution of p-mTOR and its downstream activation molecules was significantly different  
6 among the various NET types (all  $p < 0.0001$ , Figure 1). In particular, p-mTOR and p-S6K were  
7 expressed at higher levels in low-to-intermediate grade tumors (LG, corresponding to TC mets and  
8 AC) as compared to high-grade carcinomas (HG, corresponding to LCNEC and SCLC) ( $p < 0.001$   
9 and  $p = 0.027$ , respectively), whereas an opposite distribution held true for p-4EBP1 in LG and HG  
10 tumors, respectively ( $p < 0.001$ ). In the group of LG tumors, TC mets showed the highest mean H-  
11 score values for p-mTOR and p-4EBP1, albeit statistically not different as compared to the control  
12 TC and AC groups. At variance, p-S6K H-score distribution was similar in control TC and TC  
13 mets, but significantly lower in AC as compared to both TC mets and control TC ( $p < 0.001$ ).  
14 Notably, a wide dispersion of H-score values was detected within individual tumor groups (range of  
15 H-score being 0-220, 0-220, 0-170 and 0-110 for TC mets, AC, LCNEC and SCLC, respectively).  
16 In the 15 cases where metastatic tumor tissue was compared to the primary lesion, no significant  
17 differences were observed in terms of both the intensity and the percentage of positive cells for any  
18 of the three markers under investigation.

19 Western blot analysis (Figure 2) confirmed the heterogeneity of mTOR pathway activation in  
20 lung NETs. Phospho-mTOR and, more markedly, p-AKT and p-S6K proteins were expressed  
21 consistently in carcinoid samples, both typical and atypical. Interestingly, the two lung carcinoid  
22 cell lines showed opposite activation patterns thus proving to be potential models for functional  
23 tests aimed at clarifying the mechanisms of mTOR pathway activation. By contrast, in HG tumor  
24 samples both p-mTOR and p-S6K were negative or weakly positive, except for one case. In parallel,  
25 p-AKT expression was preserved, though to a generally lower extent, thus suggesting the activation  
26 of alternative AKT-mediated signalling pathways in these tumors.

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**Clinical pathological associations.** The distribution of p-mTOR, p-S6K and p-4EBP1 expression according to H-score values and clinical pathological variables is shown in Table 2.

Phospho-mTOR and its downstream effectors did not correlate with proliferation or disease stage. By contrast, in LG tumors high p-mTOR expression associated with parameters indicative of a more favorable outcome, such as negative nodal status (in AC group,  $p=0.016$ ) and disease free status ( $p=0.005$ ). Phospho-S6K followed the same association, although slightly below statistical significance, whereas p-4EBP1 did not. Moreover, p-S6K and p-4EBP1 were expressed at higher levels in small tumors, with a strong significance in LG (p-S6K,  $p=0.006$ ) and HG (p-4EBP1,  $p=0.008$ ) tumors, respectively.

No association was found between p-mTOR, p-S6K and p-4EBP1 and overall survival, in either LG or HG tumors.

**Correlation among mTOR signalling and somatostatin receptor expression.** The functional activation of mTOR signalling pathway was defined by analyzing the correlation of expression between p-mTOR and its downstream molecules. A strong positive correlation was observed between p-mTOR and its effector p-S6K. The strong correlation was maintained in LG and HG tumors when analyzed separately. Phospho-4EBP1 weakly correlated with p-mTOR but maintained a significant association with p-S6K (Table 3).

Moreover, data obtained by our group on the expression distribution of somatostatin receptor (SSTR) type 2A and 3 (Righi *et al.* 2009) in the same series, demonstrate a heterogeneous but significant progressive decrease of expression from low to high grade forms. Somatostatin receptor type 2A was over-expressed in metastatic typical carcinoids as compared to atypical carcinoids and clinically benign typical carcinoids. Furthermore a positive correlation was detected between p-mTOR (and p-S6K) and SSTR type 2A. This finding was evident both in LG and HG tumor groups ( $p=0.034$  and  $p=0.0075$ , respectively). In addition, although to a lower extent, p-mTOR correlated

1 to SSTR type 3 expression (p=0.034). By contrast p-4EBP1 did not correlate with SSTRs  
2 expression.

## 5 **DISCUSSION**

7 In the current study, we present the first evidence of activated mTOR signalling pathway in  
8 pulmonary NETs, with a specific focus on aggressive forms of these tumors, which are a challenge  
9 for the correct clinical management and could benefit from mTOR targeted therapies.

10 The functional activation of the PI3K/AKT/mTOR signalling pathway has never been  
11 extensively investigated in pulmonary or other NETs, except for indirect evidence of the expression  
12 of functionally related molecules such as PTEN (Wang *et al.* 2002), tuberous sclerosis complex  
13 (TSC) (Yao 2007), AKT (Shah *et al.* 2006) and IGF1R (von Wichert *et al.* 2000).

14 Nevertheless, the clinical interest in mTOR has increased in recent years, since the development  
15 of selective inhibitors, and several preclinical trials have been conducted to test their efficacy in  
16 different human malignancies, including NETs (Duran *et al.* 2006; Kulke 2007; Zitzmann *et al.*  
17 2007). However, controversial results in terms of clinical response to mTOR inhibitors have been  
18 obtained in NETs, possibly reflecting the heterogeneity of mTOR pathway functional status among  
19 different NET entities and within individual tumors of the same histotype.

20 A growing body of the literature regarding molecular drugs, such as EGFR tyrosine kinase  
21 inhibitors, supports the view that the selection of patients is always the fundament to benefit from  
22 these therapies at the best. Therefore, the clinical effort on the development of mTOR targeting  
23 therapies should be guided by the definition of its pathway activation status within individual NETs,  
24 also identifying specific profiles of pathogenetic and predictive interest.

25 In this context, immunohistochemistry is the most reliable, reproducible, cost-effective and  
26 clinically applicable technique to investigate large tumor series, after assuming that specific

1 antibodies against phosphorylated (active) forms of the target molecules are used through a semi-  
2 quantitative evaluation. An example supporting this point of view derives from a phase II trial on  
3 the effect of Temsirolimus (a rapamycin derivative) in advanced NE carcinomas. Although this  
4 study concluded that this agent had little activity, not warranting further single-agent evaluation in  
5 this neoplastic setting, it was clearly shown that Temsirolimus inhibited S6K phosphorylation and  
6 that higher baseline levels and lower levels after therapy of p-mTOR were predictive factors of  
7 better response (Duran *et al.* 2006).

8 Our study demonstrates that mTOR is consistently found in pulmonary NETs of different  
9 histological types, with a higher expression in low-to-intermediate grade tumors. The correlation  
10 between mTOR and its downstream molecules, confirmed also by Western Blot analysis, strongly  
11 supports the view that mTOR pathway is functionally activated in a subset of pulmonary NETs.  
12 Such correlation was stronger and more significant with p-S6K that is directly involved in mTOR  
13 signalling cascade (Guertin and Sabatini 2007) than with p-4EBP1 that, conversely, may be  
14 phosphorylated by other kinases, too (Heesom *et al.* 2001; Wang *et al.* 2003). Moreover, the  
15 heterogeneous distribution of the molecules under investigation within individual histological  
16 subtypes indicates the existence of different functional levels of the pathway, and reinforces the  
17 contention that typing different mTOR pathway-related molecules might help to correctly select  
18 patients for mTOR inhibitor-guided treatments (Meric-Bernstam and Gonzalez-Angulo 2009). In  
19 this respect, a weakness of the present study is its retrospective character and the lack of clinical  
20 correlates between mTOR and related molecule(s) expression, and the clinical response of patients  
21 to mTOR inhibitor treatments. This limitation partly reflects the current lack of standardized  
22 therapeutic approaches to the use of these drugs in the setting of clinically aggressive lung NETs.

23 Another interesting finding is the correlation that mTOR demonstrated with SSTR expression of  
24 both 2A and 3 types. Biologically, this observation seems to support the view that mTOR activity  
25 might be modulated also by SSTR in the light of experimental observations on octreotide capability  
26 to down-regulate mTOR-upstream molecules, such as PI3 Kinase and AKT, eventually leading to

1 anti-proliferative activity (Theodoropoulou *et al.* 2006). Such cross-talk between SSTRs and mTOR  
2 might explain the results of recent *in vitro* and *in vivo* studies on the anti-tumoral efficacy of  
3 combined mTOR inhibitor and octreotide treatment in NETs (Grozinsky-Glasberg *et al.* 2008;  
4 Moreno *et al.* 2008; Yao *et al.* 2008).

5 In the current tumor series, all the mTOR-related molecules failed to show a significant impact  
6 on overall survival. However, higher levels of p-mTOR and p-S6K expression were associated to  
7 more favorable clinico-pathological parameters; for example, they were found in LG tumor groups  
8 and associated with negative nodal status (in the AC group, only) or with disease free status (in LG  
9 tumor group). By contrast, 4EBP1 was unrelated to clinical pathological characteristics, suggesting  
10 activation from alternative kinase pathways other than mTOR. The literature on the prognostic role  
11 of mTOR pathway activation status in human tumors is scanty and controversial, supporting a  
12 favorable impact in some models, such as ovarian cancer (Noske *et al.* 2008), and an adverse  
13 prognostic effect in others, such as renal cell (Pantuck *et al.* 2007; Campbell *et al.* 2008), breast  
14 (Noh *et al.* 2008) and biliary tract (Herberger *et al.* 2007) carcinomas. However, no study was  
15 previously designed to investigate the prognostic implications of mTOR pathway activation players  
16 in NETs.

17 In conclusion, we first described the activation pattern of mTOR/S6K/4EBP1 signalling  
18 pathway in a large series of aggressive pulmonary NETs, also providing evidence for cross-talking  
19 with the SSTR pathway. These data support the concept that a detailed protein mapping of mTOR  
20 pathway-related molecules in lung (and possibly other) NETs may drive a more selective strategy  
21 for targeting mTOR in individual neuroendocrine tumors.

22

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2

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9

1 **REFERENCES**

2

3 Bjornsti MA & Houghton PJ 2004 The TOR pathway: a target for cancer therapy. *Nat Rev*  
4 *Cancer* 4 335-348.

5 Campbell L, Jasani B, Edwards K, Gumbleton M & Griffiths DF 2008 Combined expression  
6 of caveolin-1 and an activated AKT/mTOR pathway predicts reduced disease-free survival in  
7 clinically confined renal cell carcinoma. *Br J Cancer* 98 931-940.

8 Cappia S, Righi L, Mirabelli D, Ceppi P, Bacillo E, Ardisson F, Molinaro L, Scagliotti GV  
9 & Papotti M 2008 Prognostic role of osteopontin expression in malignant pleural mesothelioma. *Am*  
10 *J Clin Pathol* 130 58-64.

11 Dancey JE 2006 Therapeutic targets: MTOR and related pathways. *Cancer Biol Ther* 5  
12 1065-1073.

13 Duran I, Kortmansky J, Singh D, Hirte H, Kocha W, Goss G, Le L, Oza A, Nicklee T, Ho J  
14 et al. 2006 A phase II clinical and pharmacodynamic study of temsirolimus in advanced  
15 neuroendocrine carcinomas. *Br J Cancer* 95 1148-1154.

16 Duran I, Salazar R, Casanovas O, Arrazubi V, Vilar E, Siu LL, Yao J & Tabernero J 2007  
17 New drug development in digestive neuroendocrine tumors. *Ann Oncol* 18 1307-1313.

18 Garcia-Yuste M, Matilla JM & Gonzalez-Aragoneses F 2008 Neuroendocrine tumors of the  
19 lung. *Curr Opin Oncol* 20 148-154.

20 Grozinsky-Glasberg S, Franchi G, Teng M, Leontiou CA, Ribeiro de Oliveira A, Jr., Dalino  
21 P, Salahuddin N, Korbonits M & Grossman AB 2008 Octreotide and the mTOR inhibitor RAD001  
22 (everolimus) block proliferation and interact with the Akt-mTOR-p70S6K pathway in a neuro-  
23 endocrine tumour cell Line. *Neuroendocrinology* 87 168-181.

24 Guertin DA & Sabatini DM 2007 Defining the role of mTOR in cancer. *Cancer Cell* 12 9-  
25 22.



1 Heesom KJ, Gampel A, Mellor H & Denton RM 2001 Cell cycle-dependent  
2 phosphorylation of the translational repressor eIF-4E binding protein-1 (4E-BP1). *Curr Biol* 11  
3 1374-1379.

4 Herberger B, Puhalla H, Lehnert M, Wrba F, Novak S, Brandstetter A, Gruenberger B,  
5 Gruenberger T, Pirker R & Filipits M 2007 Activated mammalian target of rapamycin is an adverse  
6 prognostic factor in patients with biliary tract adenocarcinoma. *Clin Cancer Res* 13 4795-4799.

7 Huang WC, Xie Z, Konaka H, Sodek J, Zhau HE & Chung LW 2005 Human osteocalcin  
8 and bone sialoprotein mediating osteomimicry of prostate cancer cells: role of cAMP-dependent  
9 protein kinase A signaling pathway. *Cancer Res* 65 2303-2313.

10 Kulke MH 2007 New developments in the treatment of gastrointestinal neuroendocrine  
11 tumors. *Curr Oncol Rep* 9 177-183.

12 Lam JS, Pantuck AJ, Belldegrun AS & Figlin RA 2007 Protein expression profiles in renal  
13 cell carcinoma: staging, prognosis, and patient selection for clinical trials. *Clin Cancer Res* 13 703s-  
14 708s.

15 Meric-Bernstam F & Gonzalez-Angulo AM 2009 Targeting the mTOR signaling network  
16 for cancer therapy. *J Clin Oncol* 27 2278-2287.

17 Mita MM, Mita AC, Chu QS, Rowinsky EK, Fetterly GJ, Goldston M, Patnaik A, Mathews  
18 L, Ricart AD, Mays T et al. 2008 Phase I trial of the novel mammalian target of rapamycin inhibitor  
19 deforolimus (AP23573; MK-8669) administered intravenously daily for 5 days every 2 weeks to  
20 patients with advanced malignancies. *J Clin Oncol* 26 361-367.

21 Moreno A, Akcakanat A, Munsell MF, Soni A, Yao JC & Meric-Bernstam F 2008  
22 Antitumor activity of rapamycin and octreotide as single agents or in combination in  
23 neuroendocrine tumors. *Endocr Relat Cancer* 15 257-266.

24 Noh WC, Kim YH, Kim MS, Koh JS, Kim HA, Moon NM & Paik NS 2008 Activation of  
25 the mTOR signaling pathway in breast cancer and its correlation with the clinicopathologic  
26 variables. *Breast Cancer Res Treat* 110 477-483.

1           Noske A, Lindenberg JL, Darb-Esfahani S, Weichert W, Buckendahl AC, Roske A, Sehouli  
2 J, Diemel M & Denkert C 2008 Activation of mTOR in a subgroup of ovarian carcinomas:  
3 correlation with p-eIF-4E and prognosis. *Oncol Rep* 20 1409-1417.

4           Pandya KJ, Dahlberg S, Hidalgo M, Cohen RB, Lee MW, Schiller JH & Johnson DH 2007  
5 A randomized, phase II trial of two dose levels of temsirolimus (CCI-779) in patients with  
6 extensive-stage small-cell lung cancer who have responding or stable disease after induction  
7 chemotherapy: a trial of the Eastern Cooperative Oncology Group (E1500). *J Thorac Oncol* 2 1036-  
8 1041.

9           Pantuck AJ, Seligson DB, Klatte T, Yu H, Leppert JT, Moore L, O'Toole T, Gibbons J,  
10 Belldegrun AS & Figlin RA 2007 Prognostic relevance of the mTOR pathway in renal cell  
11 carcinoma: implications for molecular patient selection for targeted therapy. *Cancer* 109 2257-  
12 2267.

13           Pelosi G, Volante M, Papotti M, Sonzogni A, Masullo M & Viale G 2006 Peptide receptors  
14 in neuroendocrine tumors of the lung as potential tools for radionuclide diagnosis and therapy. *Q J*  
15 *Nucl Med Mol Imaging* 50 272-287.

16           Podsypanina K, Lee RT, Politis C, Hennessy I, Crane A, Puc J, Neshat M, Wang H, Yang L,  
17 Gibbons J et al. 2001 An inhibitor of mTOR reduces neoplasia and normalizes p70/S6 kinase  
18 activity in Pten<sup>+/-</sup> mice. *Proc Natl Acad Sci U S A* 98 10320-10325.

19           Righi L, Volante M, Tavaglione V, Billè A, Daniele L, Angusti T, Inzani F, Pelosi G, Rindi  
20 G & Papotti M 2009 Somatostatin Receptor tissue distribution in lung neuroendocrine tumours: a  
21 clinicopathologic and immunohistochemical study of 218 “clinically aggressive” cases. *Annals of*  
22 *Oncology* in press.

23           Shah T, Hochhauser D, Frow R, Quaglia A, Dhillon AP & Caplin ME 2006 Epidermal  
24 growth factor receptor expression and activation in neuroendocrine tumours. *J Neuroendocrinol* 18  
25 355-360.

1 Srirajaskanthan R, Toumpanakis C, Karpathakis A, Marelli L, Quigley AM, Dusmet M,  
2 Meyer T & Caplin ME 2008 Surgical management and palliative treatment in bronchial  
3 neuroendocrine tumours: A clinical study of 45 patients. *Lung Cancer*.

4 Theodoropoulou M, Zhang J, Laupheimer S, Paez-Pereda M, Erneux C, Florio T, Pagotto U  
5 & Stalla GK 2006 Octreotide, a somatostatin analogue, mediates its antiproliferative action in  
6 pituitary tumor cells by altering phosphatidylinositol 3-kinase signaling and inducing *Zac1*  
7 expression. *Cancer Res* 66 1576-1582.

8 Travis WD, Brambilla E, Muller-Hermelink HK & Harris CC 2004 *Tumours of the Lung,*  
9 *Pleura, Thymus and Heart*. Lyon: IARC Press.

10 Vignot S, Faivre S, Aguirre D & Raymond E 2005 mTOR-targeted therapy of cancer with  
11 rapamycin derivatives. *Ann Oncol* 16 525-537.

12 von Wichert G, Jehle PM, Hoeflich A, Koschnick S, Dralle H, Wolf E, Wiedenmann B,  
13 Boehm BO, Adler G & Seufferlein T 2000 Insulin-like growth factor-I is an autocrine regulator of  
14 chromogranin A secretion and growth in human neuroendocrine tumor cells. *Cancer Res* 60 4573-  
15 4581.

16 Wang L, Ignat A & Axiotis CA 2002 Differential expression of the PTEN tumor suppressor  
17 protein in fetal and adult neuroendocrine tissues and tumors: progressive loss of PTEN expression  
18 in poorly differentiated neuroendocrine neoplasms. *Appl Immunohistochem Mol Morphol* 10 139-  
19 146.

20 Wang X, Li W, Parra JL, Beugnet A & Proud CG 2003 The C terminus of initiation factor  
21 4E-binding protein 1 contains multiple regulatory features that influence its function and  
22 phosphorylation. *Mol Cell Biol* 23 1546-1557.

23 Yao JC 2007 Neuroendocrine tumors. Molecular targeted therapy for carcinoid and islet-cell  
24 carcinoma. *Best Pract Res Clin Endocrinol Metab* 21 163-172.

25 Yao JC, Phan AT, Chang DZ, Wolff RA, Hess K, Gupta S, Jacobs C, Mares JE, Landgraf  
26 AN, Rashid A et al. 2008 Efficacy of RAD001 (everolimus) and octreotide LAR in advanced low-

1 to intermediate-grade neuroendocrine tumors: results of a phase II study. *J Clin Oncol* 26 4311-  
2 4318.

3 Zitzmann K, De Toni EN, Brand S, Goke B, Meinecke J, Spottl G, Meyer HH &  
4 Auernhammer CJ 2007 The novel mTOR inhibitor RAD001 (everolimus) induces antiproliferative  
5 effects in human pancreatic neuroendocrine tumor cells. *Neuroendocrinology* 85 54-60.

6

1 **FIGURE LEGENDS**

2

3 **Figure 1.** Immunohistochemical distribution of p-mTOR, p-S6K and p-4EBP1 in lung NETs.

4 Upper panels illustrate an intense p-mTOR and p-S6K as well as a weak p-4EBP1 expression in a  
5 case of TC mets as compared to a case of LCNEC (middle panels) showing the opposite features. In  
6 lower panels the histograms of mTOR related molecules distribution are represented (with levels of  
7 significance for overall differences in black, for LG as compared to HG tumors in red, and for  
8 individual groups as compared to control TC at the top of each column; ns: not significant; \*:  
9  $p < 0.01$ ; \*\*:  $p < 0.001$ ). Abbreviations: TC mets: typical carcinoids with lymph node metastases; AC:  
10 atypical carcinoids; LCNEC: large cell neuroendocrine carcinoma; SCLC: small cell lung  
11 carcinoma; LG tumors: low grade tumors, including typical carcinoids with metastases and atypical  
12 carcinoids; HG tumors: high grade carcinomas, including large and small cell neuroendocrine  
13 carcinomas.

14

15 **Figure 2:** Western blot analysis of 15 cases of lung NETs and 4 lung NET cell lines.

16 Abbreviations: TC: typical carcinoid; AC: atypical carcinoid; LCNEC: large cell neuroendocrine  
17 carcinoma; SCLC: small cell lung carcinoma; LG: low grade; HG: high grade carcinomas.

1 **Table 1.** Clinicopathologic features of 218 aggressive pulmonary neuroendocrine tumors (Righi et  
 2 al, 2009).

	<b>TC mets (#24)</b> <i>No.a.</i>		<b>AC (#73)</b> <i>No.a.</i>		<b>LCNEC (#60)</b> <i>No.a.</i>		<b>SCLC (#61)</b> <i>No.a.</i>	
<b>Sex</b>	24		73		60		61	
M		12		42		53		49
F		12		31		7		12
<b>Age (y)</b>	24		73		60		61	
Range		15-78		11-77		35-87		44-84
Mean		48		55		64		65
Median		49		57		65		66
<b>Primary tumour size (mm)</b>	23		62		51		47	
≤10		1		6		2		0
11-29		14		24		16		15
≥30		8		32		33		32
Mean		25		32		42		38
<b>Ki67 (%)</b>	18		49		39		44	
Mean		3		16		70		76
Range		0,3-8		1-70		30-90		40-95
<b>Nodal status</b>	23		61		49		57	
N0		0		32		30		22
N1		16		16		11		14
N2-3		7		13		8		21
<b>Stage</b>	23		57		50		51	
1A-1B		0		13-17		9-14		9-11
2A-2B		9-6		6-8		1-12		3-9
3A-3B		8-0		9-4		7-3		15-2
4		0		0		4		2
<b>Follow-up (mos)</b>	24		72		58		61	
Follow up time:								
range		2-175		1-215		1-214		1-120
mean		47		57		41		27
Mean OS		<i>n.r</i>		122		30		23
<b>Disease status</b>	24		72		58		61	
NED/DOC		22		47		20		21
AWD		2		5		0		0
DOD		0		20		38		40
<b>Site of distant metastases</b>		Liv: 1 Lu: 1		Adr: 1 BM: 1 Bo: 3 CNS: 1 Liv: 5 Lu: 1 Med: 2 Ov:1 Pc:1 Thy:1		Bo: 4 ChW: 1 CNS: 1 Liv: 3 Lu: 3		Adr: 1 AxLN:1 Bo: 6 ChW: 1 CNS: 7 Liv: 9 Lu: 6 Med: 3

1 Abbreviations: TC mets: typical carcinoid with metastases; AC: atypical carcinoid; LCNEC: large  
2 cell neuroendocrine carcinoma; SCLC: small cell lung carcinoma; No.a.= number of available  
3 cases; M: male; F: female; RUL: right upper lobe; RML: right medium lobe; RIL: right inferior  
4 lobe; LUL: left upper lobe; LIL: left inferior lobe; n.a.= not applicable; mos= months; NED: not  
5 evidence of disease; DOC: death of other causes; AWD: alive with disease; DOD: death of disease;  
6 OS: overall survival; nr: not reached; Adr: adrenal gland; AxLN: axillary lymph node; BM: bone  
7 marrow; Bo: bone; ChW: chest wall; CNS: central nervous system; Liv: liver; Lu: lung; Med:  
8 mediastinum; Ov: ovary; Pc: pancreas; Thy: thyroid.

9

10

1 **Table 2.** Distribution of the p-mTOR, p-S6K and p-4EBP1 expression levels according to clinical  
 2 and pathological variables in 218 aggressive pulmonary neuroendocrine tumors.

	<b>p-mTOR (mean H-s)</b>	<i>p</i>	<b>p-S6K (mean H-s)</b>	<i>p</i>	<b>p-4EBP1 (mean H-s)</b>	<i>p</i>
<b><u>Size (mm)</u></b>						
<b>LG tumors (#97)</b>						
≤30	68.5	0.204	63.6	<b>0.006</b>	87.2	0.06
>30	56.3		29.7		52.8	
<b>HG carcinomas (#121)</b>						
≤30	26.5	0.53	33.7	0.22	139.3	<b>0.008</b>
>30	23.1		19.3		95.8	
<b><u>Ki-67*</u></b>						
<b>TCmets (#18)</b>						
≤2	71.5	0.89	96	0.89	74	0.63
>2	72.5		96.3		107.5	
<b>AC (#49)</b>						
≤10	65.7	0.2	40	0.64	72.8	0.19
>10	30.2		29.5		52.6	
<b>HG carcinomas (#83)</b>						
≤75	13.7	0.59	24.9	0.23	102.5	0.36
>75	15.1		28.5		118.3	
<b><u>Nodal status</u></b>						
<b>AC (#61)</b>						
N0	81.2	<b>0.016</b>	48.44	0.09	77.0	0.33
N+	33.4		23.45		53.8	
<b>HG carcinomas (#106)</b>						
N0	25.4	0.62	27.8	0.25	112.8	0.97
N+	17.4		19.7		114.8	
<b><u>Clinical Stage (TNM 2002)</u></b>						
<b>LG tumors (#96)</b>						
Stages 1-2	62.5	0.80	44.6	0.75	64.8	0.96
Stages 3-4	61.2		46.7		64.3	
<b>HG carcinomas (#101)</b>						
Stages 1-2	21.3	0.29	23.8	0.82	115.7	0.89
Stages 3-4	24.1		21.7		114.2	
<b><u>Vital status</u></b>						
<b>LG tumors (#96)</b>						
NED/DOC	71.9	<b>0.005</b>	53.6	0.06	75	0.96
AWD/DOD	31		40		83	
<b>HG carcinomas (#119)</b>						
NED/DOC	21.7	0.89	28.7	0.49	122.6	0.48
AWD/DOD	21.9		24.9		111.8	



<b>Mean Survival (months)</b>						
<b>LG tumors (#96)</b>						
low	nr	0.63	104	0.152	nr	0.81
high	122		nr		122	
<b>HG carcinomas (#119)</b>						
low	25	0.44	34	0.258	28	0.63
high	28		23		27	

1 **Abbreviations:** H-s: H-score; TC mets: typical carcinoids with metastases; AC: atypical carcinoids;

2 LG tumors: low grade tumors, including typical carcinoids with metastases and atypical carcinoids;

3 HG carcinomas: high grade carcinomas, including large and small cell neuroendocrine carcinomas;

4 \*: cut off values of Ki-67 correspond to medians in each group; NED: not evidence of disease;

5 DOC: death for other causes; AWD: alive with disease; DOD: death of disease; nr: not reached.

6

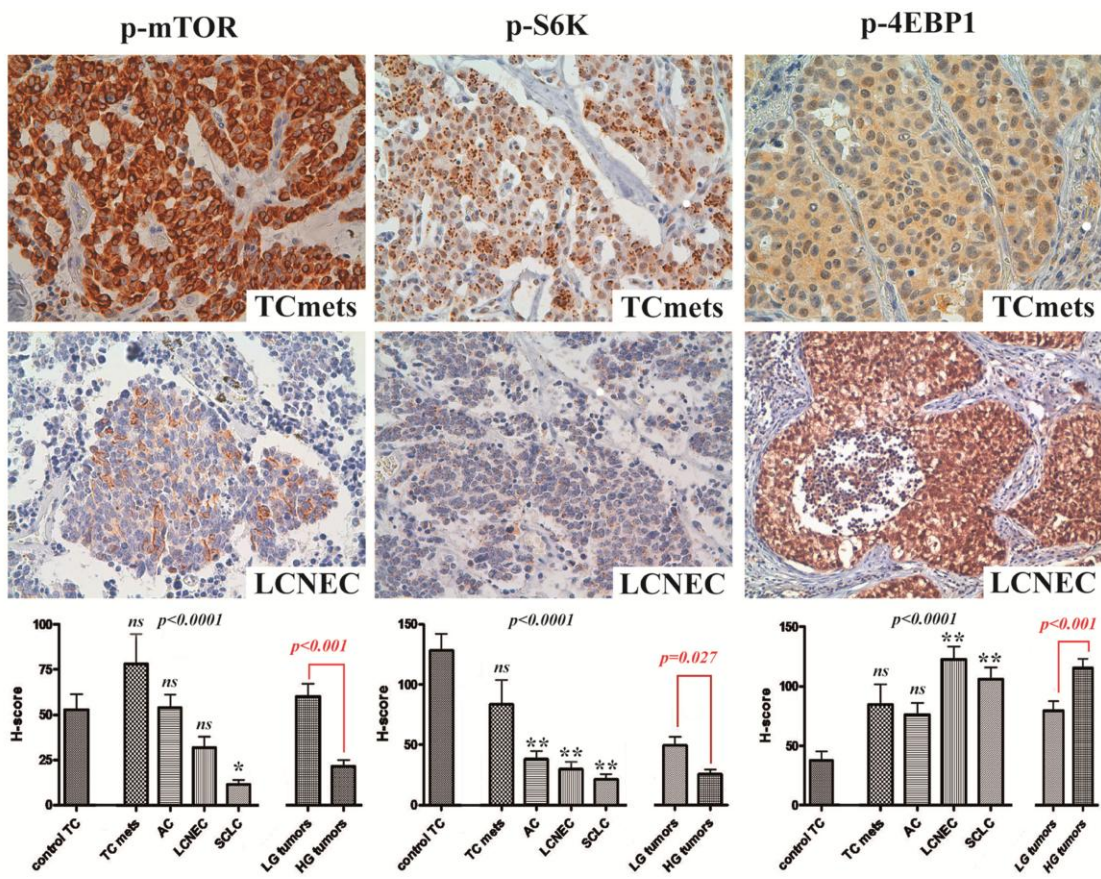
1 **Table 3.** Correlation of p-mTOR, p-S6K and 4EBP-1 with somatostatin receptors  
 2 in 218 pulmonary neuroendocrine tumors.

	<b>p-mTOR</b>	<b>p-S6K</b>	<b>p-4EBP1</b>
<b>p-mTOR</b>	-	<b>p&lt;0.0001</b> <i>r=0.458</i>	<b>p=0.0074</b> <i>r=0.181</i>
<b>p-S6K</b>	-	-	<b>p&lt;0.0001</b> <i>r=0.312</i>
<b>SSTR-2A</b>	<b>p&lt;0.0001</b> <i>r=0.271</i>	<b>p=0.046</b> <i>r=0.135</i>	p=0.7 <i>r=-0.023</i>
<b>SSTR-3</b>	<b>p=0.034</b> <i>r=0.271</i>	p=0.9 <i>r=-0.069</i>	p=0.9 <i>r=0.016</i>

14

15 **Abbreviations:** SSTR: somatostatin receptor.

1



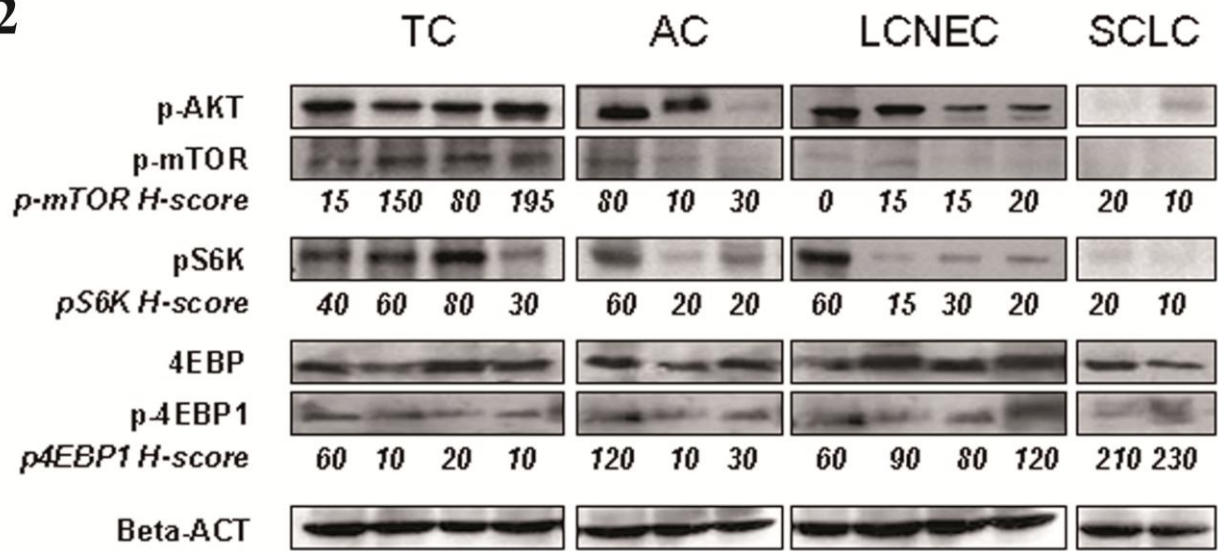
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3 **Figure 1**

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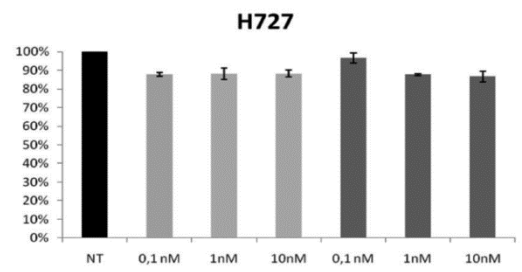
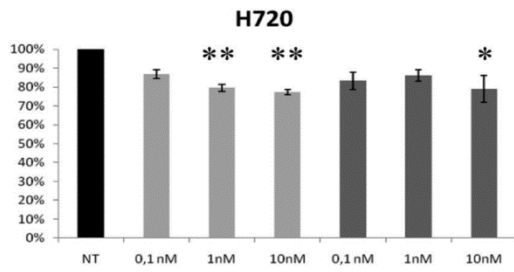
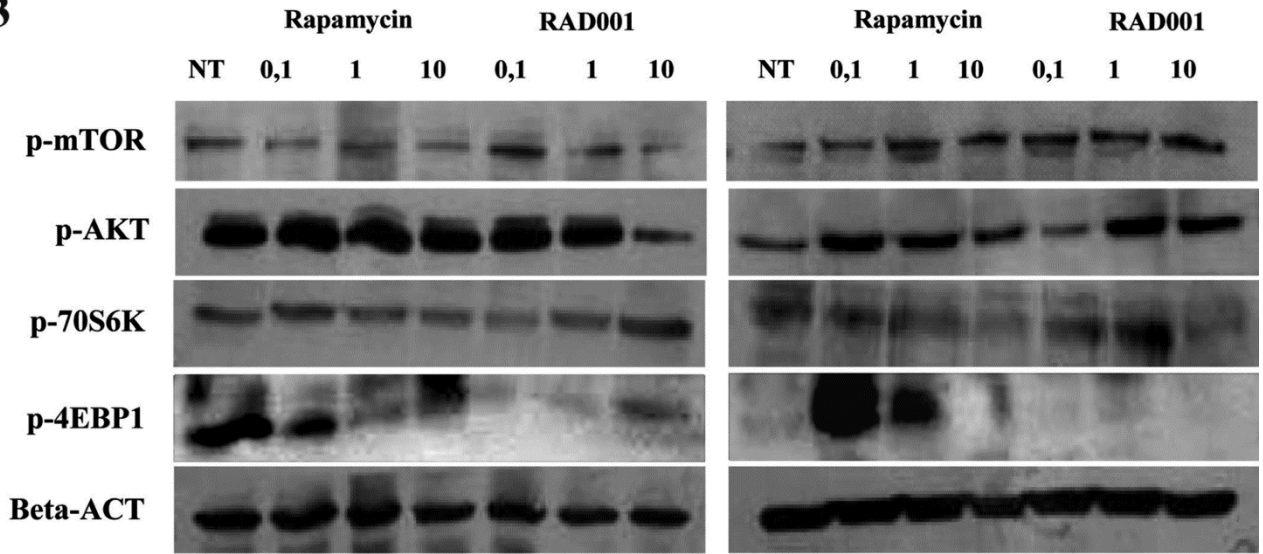


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2 **Figure 2**

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2 Figure 3