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

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Short Title: mTOR signalling pathway in lung neuroendocrine tumors.

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

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ABSTRACT

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

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

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

mTOR is a serine threonine kinase that participates in the regulation of proliferation, cell growth and apoptosis through modulation of cell cycle progression (Vignot et al. 2005). The activated (phosphorylated) mTOR kinase leads to the subsequent phosphorylation of downstream effectors: the ribosomal p70S6-kinase (S6K) and the eukaryotic initiation factor 4E-binding protein 1 (4EBP1), two key proteins that regulate translation of mRNAs into proteins required for cell cycle progression from G1 to S phase (Podsypanina et al. 2001; Dancey 2006). Recent insights revealed a significant complexity of the mTOR pathway that seems to cross talk with other well-characterized signalling cascades, thus paving the way to the use of combined therapies (Bjornsti and Houghton 2004; Guertin and Sabatini 2007; Meric-Bernstam and Gonzalez-Angulo 2009). mTOR signalling
pathway can be upstream activated - most commonly via the PI3 Kinase/AKT pathway - by receptors such as somatostatin receptors or insulin-like growth factor receptor 1 or by loss of inhibiting molecules, such as PTEN (von Wichert et al. 2000; Wang et al. 2002). Rapamycin (Sirolimus, Wyeth, Philadelphia, PA) and its derivates are immunosuppressive macrolides that specifically block mTOR signalling and have been shown to possess anti-proliferative activity in a variety of malignancies both in vitro (Zitzmann et al. 2007) and in phase II clinical trials (Yao et al. 2008). Inhibition of mTOR prevents phosphorylation of S6K, 4EBP1 and, indirectly, other proteins involved in the transcription and cell cycle control, leading to G1 phase cell growth arrest.

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

Despite all the above pre-clinical and clinical studies on the anti-neoplastic efficacy of mTOR inhibitors in a variety of tumors, data on the activation status of mTOR signalling cascade in pulmonary NET are still lacking. In this respect, a detailed protein expression map of mTOR pathway-related molecules in lung NET, could not only define specific expression patterns predictive of clinical response, as suggested for other malignancies (Lam et al. 2007), but also
investigate the prognostic implications of these molecules. Therefore, aim of this study was to evaluate the expression of activated mTOR related proteins in a large series of pulmonary NET - with special reference to clinically malignant cases.

MATERIALS AND METHODS

Case selection. Eight hundred eighty three surgically resected NETs of the lung were recorded between 1989 and 2007 in the pathology files of the Universities of Turin and Parma and the European Institute of Oncology of Milan (467, 188 and 217 cases, respectively). Among them, a series of 218 clinically malignant lesions (129 cases from Turin, 40 from Parma and 49 from Milan) was collected, including 24 typical carcinoids (TC) with lymph node metastases at the time of the diagnosis (TC mets), 73 atypical carcinoids (AC) with or without lymph node metastases, 60 large cell neuroendocrine carcinomas (LCNEC) and 61 small cell carcinomas (SCLC). Pathological samples corresponded to primaries in all but 15 cases, where lymph node metastases were the only available material. In 15 cases, primary tumor and corresponding lymph node metastasis was analyzed. All cases were classified according to the last 2004 WHO Classification on Lung Tumors (Travis et al. 2004) and the clinical and pathological characteristics collected elsewhere (Righi et al. 2009) and including sex, age, primary tumour size, Ki-67 labeling index (expressed as the percentage of positive cells in highest labeling areas), nodal status, stage, follow up and site of distant metastases were detailed in Table 1. Forty consecutive non metastatic TC were raised from the files of the University of Turin to be used as control group for baseline expression of the markers under evaluation. All cases were anonymized by a pathology staff member not involved in the study. Clinical data were compared and analysed through coded data, only. The study was approved by the institutional review board of the Hospital.
**Immunohistochemistry.** Immunohistochemistry was performed using monoclonal antibodies against Ser2448-phospho-mTOR (p-mTOR, rabbit 49F9, diluted 1/100), Thr389-phospho-p70S6K, (p-S6K, mouse 1A5, diluted 1/400) and Thr37/46-phospho-4EBP1 (p-4EBP1, rabbit 236B4, diluted 1/300); all antibodies were purchased from Cell Signalling Technologies, Beverly, MA. Five micron-thick paraffin sections were collected onto charged slides, deparaffinized and re-hydrated in water. After antigen retrieval in pH 6.0 citrate buffer for 5 minutes at 125°C in a pressure cooker, the relevant primary antibodies were incubated overnight at 4°C. Immunoreactions were revealed by a biotin-free dextran-chain detection system (Envision, DakoCytomation, Glostrup, Denmark), and developed using 3’-3’-diaminobenzidine as the chromogen. The specificity of all reactions was validated in parallel control sections omitting the primary antibodies for each immunohistochemical run.

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

**Western Blot analysis.** Four lung neuroendocrine tumor cell lines (from typical - H727 - and atypical - H720 - carcinoids, and from small cell carcinomas, H69 and H526) were available from ATCC (Manassan, VA, USA). In addition, 14 frozen lung NET samples, not included in the present
series of 218 cases, were available from the tissue bank of the Pathology Unit at the University of Turin.

All samples were homogenized and lysed in TNE lysis buffer supplemented with 1% protease inhibitor cocktail (Complete, Roche Diagnostic Corporation, IN). The protein concentration was evaluated using BCA protein assay Kit (Pierce, Milwaukee, WI), and 50 micrograms of protein were resolved in 8% SDS-PAGE and transferred to nitrocellulose membranes for each experiment. The membrane blots were blocked for 1h with 5% BSA in TBS-Tween 0.1% and incubated overnight at 4°C with primary antibody: anti-p-AKT (Thr 308, 1:1000), anti-p-mTOR (Ser2884, 1:1000), anti-p-p70S6K (Thr 389, 1: 2000) (all from Cell Signalling Technology, Beverly, MA). Anti-β-Actin (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) was used as the loading control. Immunoreactive proteins were visualized using horseradish peroxidase–conjugated anti-mouse or anti-rabbit antibody (1:3,000 and 1:1,000, respectively) and Enhanced Chemiluminescence (ECL) (Amersham, Biosciences, Piscataway, NJ) as the substrate.

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

**RESULTS**

**Distribution of mTOR signalling molecules in lung NETs.** Immunohistochemical staining for p-mTOR and p-4EBP1 provided the expected cytoplasmic pattern, while p-S6K showed either a cytoplasmic perinuclear dot-like or a diffuse nuclear pattern of staining.
In peri-tumoral non neoplastic parenchyma, a weak p-mTOR, p-S6K and p-4EBP1 immunoreactivity was observed in normal bronchial epithelium and endothelia. Alveolar histiocytes were also reactive for p-S6K and p-4EBP1, whereas a strong p-mTOR immunoreactivity was detected in reactive alveolar epithelial cells at the periphery of the tumors (data not shown).

Distribution of p-mTOR and its downstream activation molecules was significantly different among the various NET types (all p<0.0001, Figure 1). In particular, p-mTOR and p-S6K were expressed at higher levels in low-to-intermediate grade tumors (LG, corresponding to TC mets and AC) as compared to high-grade carcinomas (HG, corresponding to LCNEC and SCLC) (p<0.001 and p=0.027, respectively), whereas an opposite distribution held true for p-4EBP1 in LG and HG tumors, respectively (p<0.001). In the group of LG tumors, TC mets showed the highest mean H-score values for p-mTOR and p-4EBP1, albeit statistically not different as compared to the control TC and AC groups. At variance, p-S6K H-score distribution was similar in control TC and TC mets, but significantly lower in AC as compared to both TC mets and control TC (p<0.001). Notably, a wide dispersion of H-score values was detected within individual tumor groups (range of H-score being 0-220, 0-220, 0-170 and 0-110 for TC mets, AC, LCNEC and SCLC, respectively).

In the 15 cases where metastatic tumor tissue was compared to the primary lesion, no significant differences were observed in terms of both the intensity and the percentage of positive cells for any of the three markers under investigation.

Western blot analysis (Figure 2) confirmed the heterogeneity of mTOR pathway activation in lung NETs. Phospho-mTOR and, more markedly, p-AKT and p-S6K proteins were expressed consistently in carcinoid samples, both typical and atypical. Interestingly, the two lung carcinoid cell lines showed opposite activation patterns thus proving to be potential models for functional tests aimed at clarifying the mechanisms of mTOR pathway activation. By contrast, in HG tumor samples both p-mTOR and p-S6K were negative or weakly positive, except for one case. In parallel, p-AKT expression was preserved, though to a generally lower extent, thus suggesting the activation of alternative AKT-mediated signalling pathways in these tumors.
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 an 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
to SSTR type 3 expression (p=0.034). By contrast p-4EBP1 did not correlate with SSTRs expression.

DISCUSSION

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

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

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

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

In this context, immunohistochemistry is the most reliable, reproducible, cost-effective and clinically applicable technique to investigate large tumor series, after assuming that specific
antibodies against phosphorylated (active) forms of the target molecules are used through a semi-
quantitative evaluation. An example supporting this point of view derives from a phase II trial on
the effect of Temsirolimus (a rapamycin derivative) in advanced NE carcinomas. Although this
study concluded that this agent had little activity, not warranting further single-agent evaluation in
this neoplastic setting, it was clearly shown that Temsirolimus inhibited S6K phosphorylation and
that higher baseline levels and lower levels after therapy of p-mTOR were predictive factors of
better response (Duran et al. 2006).

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

Another interesting finding is the correlation that mTOR demonstrated with SSTR expression of
both 2A and 3 types. Biologically, this observation seems to support the view that mTOR activity
might be modulated also by SSTR in the light of experimental observations on octreotide capability
to down-regulate mTOR-upstream molecules, such as PI3 Kinase and AKT, eventually leading to
anti-proliferative activity (Theodoropoulou et al. 2006). Such cross-talk between SSTRs and mTOR might explain the results of recent in vitro and in vivo studies on the anti-tumoral efficacy of combined mTOR inhibitor and octreotide treatment in NETs (Grozinsky-Glasberg et al. 2008; Moreno et al. 2008; Yao et al. 2008).

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

In conclusion, we first described the activation pattern of mTOR/S6K/4EBP1 signalling pathway in a large series of aggressive pulmonary NETs, also providing evidence for cross-talking with the SSTR pathway. These data support the concept that a detailed protein mapping of mTOR pathway-related molecules in lung (and possibly other) NETs may drive a more selective strategy for targeting mTOR in individual neuroendocrine tumors.
**Declaration of interest:** all Authors declare the absence of any conflict of interest.

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REFERENCES


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


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

**FIGURE LEGENDS**

**Figure 1.** Immunohistochemical distribution of p-mTOR, p-S6K and p-4EBP1 in lung NETs. Upper panels illustrate an intense p-mTOR and p-S6K as well as a weak p-4EBP1 expression in a case of TC mets as compared to a case of LCNEC (middle panels) showing the opposite features. In lower panels the histograms of mTOR related molecules distribution are represented (with levels of significance for overall differences in black, for LG as compared to HG tumors in red, and for individual groups as compared to control TC at the top of each column; ns: not significant; *: p<0.01; **: p<0.001). Abbreviations: TC mets: typical carcinoids with lymph node metastases; AC: atypical carcinoids; LCNEC: large cell neuroendocrine carcinoma; SCLC: small cell lung carcinoma; LG tumors: low grade tumors, including typical carcinoids with metastases and atypical carcinoids; HG tumors: high grade carcinomas, including large and small cell neuroendocrine carcinomas.

**Figure 2:** Western blot analysis of 15 cases of lung NETs and 4 lung NET cell lines. Abbreviations: TC: typical carcinoid; AC: atypical carcinoid; LCNEC: large cell neuroendocrine carcinoma; SCLC: small cell lung carcinoma; LG: low grade; HG: high grade carcinomas.
Table 1. Clinicopathologic features of 218 aggressive pulmonary neuroendocrine tumors (Righi et al, 2009).

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Abbreviations: TC mets: typical carcinoid with metastases; AC: atypical carcinoid; LCNEC: large cell neuroendocrine carcinoma; SCLC: small cell lung carcinoma; No.a.= number of available cases; M: male; F: female; RUL: right upper lobe; RML: right medium lobe; RIL: right inferior lobe; LUL: left upper lobe; LIL: left inferior lobe; n.a= not applicable; mos= months; NED: not evidence of disease; DOC: death of other causes; AWD: alive with disease; DOD: death of disease; OS: overall survival; nr: not reached; Adr: adrenal gland; AxLN: axillary lymph node; BM: bone marrow; Bo: bone; ChW: chest wall; CNS: central nervous system; Liv: liver; Lu: lung; Med: mediastinum; Ov: ovary; Pc: pancreas; Thy: thyroid.
Table 2. Distribution of the p-mTOR, p-S6K and p-4EBP1 expression levels according to clinical and pathological variables in 218 aggressive pulmonary neuroendocrine tumors.

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<tr>
<th></th>
<th>p-mTOR (mean H-s)</th>
<th>p</th>
<th>p-S6K (mean H-s)</th>
<th>p</th>
<th>p-4EBP1 (mean H-s)</th>
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<td>high 122</td>
<td>28 0.63 122</td>
<td>28 0.63 28 0.63</td>
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</table>

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

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<tr>
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<th>p-4EBP1</th>
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<td>p&lt;0.001</td>
<td>p=0.0074</td>
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<tr>
<td>p-S6K</td>
<td>r=0.458</td>
<td>r=0.181</td>
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<tr>
<td>SSTR-2A</td>
<td>p&lt;0.0001</td>
<td>p=0.046</td>
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<td>r=0.271</td>
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<td>r=0.271</td>
<td>r=-0.069</td>
<td>r=0.016</td>
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</table>

**Abbreviations:** SSTR: somatostatin receptor.
Figure 1
Figure 2
Figure 3