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mTOR is Frequently Active in GH-Secreting Pituitary Adenomas without Influencing their Morphopathological Features

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Abstract Initiating factors and mechanisms of tumor formation are poorly understood in nonfamilial pituitary adenomas. Alteration of intracellular pathways is an underlying event in numerous neoplasms. Among them, excessive activation of mammalian target of rapamycin (mTOR) pathway and its two main regulators, Akt and Erk, has been detected frequently in solid tumors. This study tests the activation of mTOR pathway in pituitary adenomas and its influence on their morphopathological features. Fifty-three pituitary adenomas were fresh frozen after surgery and analyzed by western blotting using phospho-specific antibodies. The impact of Akt and Erk activation on mTOR pathway was assessed in five primary cultures derived from the excised adenomas using selective kinase inhibitors. Statistical correlations of size, volume, Ki-67 %, Knosp's grading, and somatostatin receptor (SSTR) expression with the activation of mentioned kinases was performed. GHomas showed the highest frequency (71 %) and level of mTOR pathway activity comparing to other adenomas (33 %). No significant correlation was found between mTOR activation and any of the morphopathological features in the studied samples. mTOR kinase phosphorylation was independent of Erk and Akt in primary cultures. Erk activity was significant in all types of adenomas but was the highest in control samples. Its phosphorylation correlated inversely with the Knosp's grading in nonfunctional pituitary adenomas and directly with somatostatin receptor subtype 2 A expression in GHomas. Presented data point to the noteworthy mTOR activity in GHomas. However, the lack of correlation with morphopathological features, its independence of Erk and Akt phosphorylation, and high level of Erk activity in control pituitary necessitate further research for clarifying the role of these pathways in pituitary adenomas.

Keywords Pituitary adenoma · mTOR · Erk · Akt · Immunohistochemistry

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Introduction

Pituitary adenomas (PAs) are a diverse group of benign tumors arising from the anterior pituitary gland. They are the third most common intracranial neoplasms after meningiomas and gliomas, accounting for approximately 15 % of all intracranial tumors [1]. PAs are grossly characterized by their hormonal activity as functional or nonfunctional. Depending on the produced hormone, functional adenomas are further classified as GHoma, PRLoma, ACTHoma, and TSHoma. Although nonfunctional pituitary adenomas (NFPAs) often originate form gonadotropin-secreting cells, they show no hormonal excretion. Current World Health

Organization (WHO) classification of PAs is based on immunostaining and transmission electron microscopy [2, 3]. Ki-67 protein expression in nuclei of proliferating cells is also being assessed routinely in excised PAs as an indicator of tumor growth [4, 5]. Clinically, PAs result in syndromes associated with an excessive, autonomous excretion of hormones, pituitary insufficiency, or symptoms related to their mass effect and infiltration of surrounding tissues. Due to the variety of manifestations caused by pituitary adenomas, they are an interdisciplinary relevant healthcare issue. However, most of PAs are asymptomatic or display nonspecific, insidious signs being identified as incidental findings. In a recent meta-analysis, an overall prevalence of pituitary adenomas reaching 16.7 % in general population was estimated (14.4 % in autopsy and 22.5 % in radiological studies) [6]. Thus, the need for identifying factors that cause the formation of adenomas and, in the next step, mark out the symptomatic cases from asymptomatic ones seems relevant.

Tumorigenic events in PA are only partly understood. Some pituitary adenomas are components of genetic familial syndromes caused by known mutations including McCune Albright syndrome and multiple endocrine neoplasia [7–9]. However, the majority of PAs are sporadic cases with a rather elusive initiating aberration and the aforementioned mutations or mutations in well-known tumor suppressor genes or oncogenes are very rarely found [10]. Thus, studies aiming to explain the pathophysiology of pituitary adenomas have taken up many directions. One of them is the search for hyperactivation of intracellular pathways. Among them, mammalian target of rapamycin (mTOR) pathway has been recently emerging as a new target. It regulates metabolism, growth, and proliferation in normal cells and has been shown to play a critical role in proliferation, survival, and drug resistance in many human cancers. mTOR, a serine-threonine kinase, plays a central role in this pathway as it integrates stimuli from growth factors, nutrients, and energy status [11]. Although mTOR cascade has often been named PI3K/Akt/mTOR, it is clear now that besides its canonical activator, Akt, there is a line of other kinases located upstream of mTOR, including the RSK kinase, Ras/MEK/ERK pathway, AMPK (engaged in energy sensing pathway) or hypoxia response pathway (hypoxia induced *REDD1* gene). Being situated in the crossroad of these pathways, mTOR acts as a sensor of parameters that define the wellbeing of the cell, determining whether the cell is able to grow and proliferate [12]. Among these activators, Ras/MEK/ERK is of special interest in pituitary pathophysiology. Upregulation of its components at each level has been shown in all types of PAs comparing to normal pituitary tissue [10, 13]. Apart from that, higher levels of Akt activity has been also demonstrated in PAs, putting forward the mTOR pathway as a potential player in pituitary tumorigenesis. The two mentioned regulatory kinases for mTOR, Akt, and Erk, displayed the highest levels of activation in NFPAs [13–15]. Their activation in this type of PA was also proposed as screening markers for identifying patients with higher risk of recurrence [16]. The phosphorylation of mTOR kinase itself in PAs was assessed directly in one study. However, it failed to detect any differences between PA and normal pituitary tissue despite strong activation of Akt and Erk [13]. In this study, we aimed to assess the activity of mTOR and Erk in pituitary adenomas at the phosphorylation level using western blotting method in different types of PAs. mTOR and Ras/MEK/ERK pathway has been extensively studied in many tumors and proved to be hyperactive [17–19]. Phosphospecific antibodies against Erk allow for detection of active form of the kinase that has the ability to phosphorylate and activate mTOR. mTOR activity was demonstrated through detection of phosphorylation of S6 ribosomal protein (S6rp). S6rp is one of the main effectors of mTOR and is often used as the read out of its activity [20–22]. Activation of S6rp exclusively by mTOR was proved in primary cultures using specific kinase inhibitors. Our findings were correlated with clinical and histological data in each adenoma in an attempt to anticipate the effect of the activation of mTOR and Erk kinase on PA features.

Materials and Methods

Patients

In total, 53 patients who underwent elective transsphenoidal surgery (26 women and 27 men) were enrolled in this study. Patients' age ranged between 28 and 83 (mean age, 54.6± 15.7). Participants gave written informed consent for the protocol, which was approved by the local ethical committee. Tissue obtained during surgery was immediately flash-frozen for further analysis. Collected samples included: 14 GHomas, 33 NFPAs, and five ACTHomas (clinical and morphopathological details of each case are given in Electronic Supplementary Material (ESM) 1). In several cases, concurrently with the frozen sampling, tissue was immediately processed in attempt to establish primary cultures, as described below. Control pituitary tissue was obtained from five cases of micro-ACTHomas where the tumor was not visualized clearly on magnetic resonance imaging (MRI). The suspected tumor was excised with the surrounding tissues. After the exploratory surgery, resected tissue was examined in the laboratory, where tissue of the non-adenomatous appearance was separated. This was confirmed in histopathological examination; therefore, in further studies, these tissue samples were used as the pituitary control.

Immunostaining and Histopathology

Histopathological diagnosis was based on conventional light microscopy and immunohistochemical staining for pituitary hormones in accordance with the WHO classification of tumors of the endocrine system [3]. Briefly, material was fixed in 10 % formalin, embedded in paraffin and routinely stained with hematoxylin and eosin. Immunohistochemical staining was performed on paraffin-embedded specimens according to the labeled EnVision Flex Visualization System (Dako, Denmark) with DAB (3,3′-diaminobenzidine) as chromogen, using antibodies against: prolactin (PRL, dilution 1:200), growth hormone (GH, dilution 1:500), ACTH (dilution 1:500), β-TSH (dilution 1:200), β-FSH (dilution 1:500), β-LH (dilution 1:500) (all from LAB VISION, Thermo Fisher Scientific, USA), the glycoprotein α-subunit (dilution 1:100, Novocastra, UK), Ki-67 (MIB1, dilution 1:100; Dako, Denmark) and phosphorylated S6 ribosomal protein (Ser235/236; dilution 1:400, Cell Signaling Technology, USA). MIB-1 labeling index was routinely established in all pituitary adenoma specimens. In GHomas, antibodies against somatostatin receptor proteins: 2A type (sstr2A; SS-800) and 5 type (sstr5; SS-890) were also used (Biotrend, Germany).

Electron Microscopy

Small pieces of each GHoma were fixed in glutaraldehyde, postfixed in osmium tetroxide, and embedded in Epon. Ultrathin sections counterstained with uranyl acetate and lead citrate were examined with a Philips CM120 BioTWIN electron microscope.

Protein Extraction and Western Blotting

Protein lysates were prepared from approximately 5 mm^3 of tissue. Samples were pesteled on ice in 200 μl of chilled RIPA buffer supplemented with 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, phospatase inhibitor cocktail (PhosSTOP, Roche, Basel, Switzerland), and protease inhibitors (Complete, Roche, Basel, Switzerland). The grinded tissue was rested for 15 min on ice. Lysates were cleared by centrifugation at 12,000×*g* at 4 °C. The supernatants were assayed for protein concentration by Lowry method and stored at −80 °C until further analysis. For western blotting, 50 μg of the protein was separated on 15 % SDS-PAGE and transferred to PVDF membrane (Immobilon-P; Millipore, Watford, Herts, UK). The membrane was blocked then with 5 % non-fat dry milk in Tris-buffered saline/Tween 20 and incubated with primary antibodies overnight at 4 °C. A cocktail of primary antibodies against phospho-Akt (Ser473), phospho-Erk1/2 (Thr202/Tyr204), and phosphorylated S6 ribosomal protein (Ser235/236) was used (Cell Signaling Technology, USA). Primary antibody against eIF4E included in the cocktail was used for protein loading control according to manufacturer (Cell Signaling Technology, USA). Its stability, sufficient for this purpose, has been proven previously [23, 24]. After incubations with primary antibody cocktail, membranes were washed and incubated with secondary, HRP-labeled antibody for 45 min and, after washing, the phosphorylated proteins were detected using the western blotting luminol reagent (Santa Cruz, CA, USA) and visualized on X-ray films. The films were analyzed using Image J software (NIH, USA) [25]. The activation of each kinase was normalized to the expression of a housekeeping gene-eIF4E in the same sample and was expressed as the ratio of signal from a given phospho-protein to the signal form eIF4E.

Primary Cell Culture

Excised pituitary adenomas were processed within 3 h from the surgery. A small, 5 mm^3 fragment of adenoma was saved for protein analysis by western blotting as described above. The remaining tissue was cut into small pieces, washed in PBS and centrifuged for 5 min at 1,500×*g* three times. Cells were dispersed enzymatically in 0.5 % collagenase and 0.05 %/0.02 % trypsin/EDTA in M-199 medium for 1 h at 37 °C being vigorous shaken every 10 min. After digestion FSC was added to the final concentration of 10 % and the cell suspension was centrifuged at 800×*g* for 20 min in room temperature. The cell pellet was dispersed then in M-199 medium supplemented with 10 % fetal calf serum (FCS) and penicillin/streptomycin. Matrigel (BD, Bedford, MA, USA) was mixed with serum free cell culture medium in 1:4 ratio, poured on the plates and incubated for polymerization for 30 min in 37 °C. Primary PA cells were plated on matrigel-coated 6-well plates, and cultured in 5 % CO_2 atmosphere at 37 °C for 48 h before tests.

Drug Tests

To analyze the effect of specific kinases on mTOR activation, 20 nM rapamycin (mTOR inhibitor), 100 nM wortmanin (Akt inhibitor), and 10 μM U0126 (Erk inhibitor) were added to the cell cultures as described previously [26] (indicated are respective final concentrations of each compound). Pure solvent (DMSO; final *v*/*v* concentration 0.1 %) was added to the "untreated" control culture. To enhance test sensitivity, cells were incubated in serum-free medium for 2 h before inhibitors were used. Forty-five minutes after inhibitors were administrated, culture medium was supplemented with FCS (final concentration, 10 %). 10 min later cells were harvested and lyzed. The extraction of the protein and the WB was carried out as described above.

Tumor Invasiveness and Size Estimation

Tumor invasiveness was based on MRI imaging and intra-operative findings and expressed according to the Knosp's grading [27]. Tumor diameter and its approximate volume were calculated using MRI scans. To simplify calculations, adenoma shape was considered ellipsoid. Using ellipsoid volume formula $V=4/3\pi abc$, where a, b, and c are the three semi-axes, tumor volume was estimated.

Statistical Analysis

Statistical analysis was performed with Statistica software (Statsoft, Tulsa, USA) with the use of the Fisher exact probability test, Spearman's rank correlation coefficient, *U* Mann–Whitney test, and Kruskal–Wallis test. Statistical significance was defined at $p<0.05$.

Results

mTOR is Active in Pituitary Adenomas

The activation of mTOR was calculated as pS6rp/elF4E ratio in all tissue samples (see Table in ESM 1, which contains the detailed measurements). An adenoma was considered to have active mTOR when pS6rp/elF4E >0 (Table 1, Figs. 1 and 2).

GHoma samples had the highest frequency of mTOR activity comparing to other types of adenomas (GHoma vs NFPA $p=0.018$, GHoma vs NFPA+ACTHoma $p=0.02$ in Fisher exact probability test). Moreover, GHomas showed higher level of mTOR activity compared to NFPAs ($p=0.04$ in *U* Mann–Whitney test). In control pituitary tissue, one sample out of five had active mTOR (pS6rp/elF4E=0.085).

pErk Activity is High in all Adenoma and Control Pituitary Tissue Samples

Erk was activated in most pituitary samples, including control samples (see Table in the ESM 1, which contains the detailed measurements). The level of Erk activity, calculated as pErk/elF4E ratio, was the highest in control pituitary samples reaching statistical significance ($p=0.003$ in Kruskal–Wallis test comparing all groups of adenomas and normal pituitary tissue; Table 2, Figs. 1 and 3).

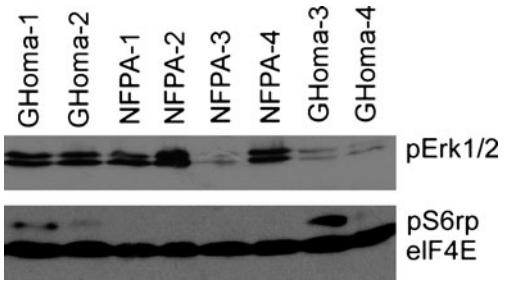

Fig. 1 mTOR and pErk1/2 in a sample of pituitary adenomas. The phosphorylation of S6 ribosomal protein (pS6rp) is used as the read out of mTOR activity. No pS6rp was detected in any of the NFPAs whilst GHoma-1 and GHoma-3 have a strong signal for it. pErk1/2 was detected in both types of adenomas and is not coupled with mTOR activity. elF4E served as loading control

Correlation Analysis of Kinases Activity and Morphopathological Features of Adenoma Samples

Correlation analysis was performed (Table 3). Additionally in NFPAs and GHomas, mTOR active and non-active adenomas were analyzed separately. The level of mTOR activity did not show any significant correlation with any of the following parameters: tumor volume, tumor largest dimension, Knosp's grading, Ki-67 %, and pErk activity in any set of samples.

In NFPAs, the activity of pErk showed a medium level of inverse correlation with Knosp's grading that reached statistical significance (*R* Spearman=−0.31, $p=0.018$). In mTOR non-active NFPAs, this correlation became even stronger(*R* Spearman=−0.48, $p=0.022$) and was not present in mTOR-active samples.

In GHomas, pErk showed a strong level of correlation with somatostatin receptor subtype 2 A (SSTR2A) expression (*R* Spearman=0.57, $p=0.04$). Among GHomas, 6/14 were densely granulated, 3/14 were sparesly granulated in electron microscopy, and 3/14 were GH+/PRL+. There were no significant differences in between GHoma types.

Table 1 The frequency of mTOR active adenomas presented as percentage of samples with active mTOR in the given type of adenoma and, in parenthesis, as number of active samples/number of analyzed samples. The data are non-normally distributed

Type	mTOR activity frequency	Median of pS6rp/elF4E	25–75 percentiles of pS6rp/elF4E	Minimum/Maximum detected pS6rp/elF4E
GHoma	71 % (10/14)	0.093	0–0.180	0/0.379
NFPA	33 % (11/33)	0	0–0.44	0/0.548
ACTHoma	33 % (2/6)	0	0–0.42	0/0.064
All adenomas	43 % (23/53)	0	0–0.143	0/0.548
Control	20 % (1/5)	0	0–0	0/0.085

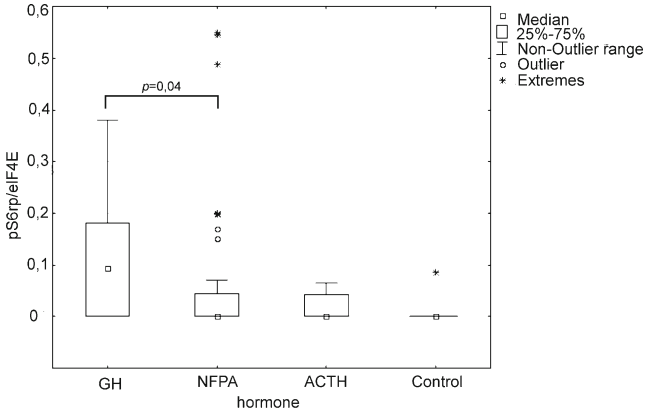

Fig. 2 mTOR activity in pituitary adenomas. mTOR kinase activity was calculated as pS6rp/elF4E ratio. The control group consisted of five specimens, with a zero value for pS6rp/elF4E ratio in four of them. GHomas had the highest level of mTOR activity, that reached statistical significance in comaprison to NFPA (p=0.04)

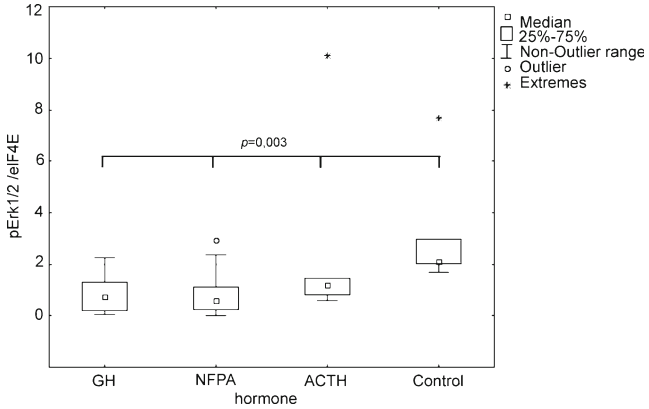

Fig. 3 pErk1/2 activity in pituitary adenomas. Erk kinase activity was calculated as pErk1/2/elF4E ratio. Control pituitary tissue had the highest level of pErk1/2 (p=0.003)

mTOR Activation in Primary Cultures of Pituitary Adenomas can be Mediated by Mechanisms Other Than Akt and Erk Kinases

Primary cultures form mTOR active GHoma, NFPA, and ACTHoma were setup on matrigel. All derived primary cell cultures displayed identical pattern of activation of mTOR, Erk, and Akt. When selective inhibitors were used, all cell lines showed mTOR inhibition in response to rapamycin while inhibition of Erk did not affect mTOR pathway despite the complete inhibition of targeted kinase. Also, inhibition of PI3K/Akt in ACTHoma line, did not modulate mTOR activation. Two adenomas with primarily non-active mTOR were also cultured. Despite the lack of mTOR activity in the tumor, cell cultures exhibited high level of S6rp phosphorylation with a pattern of kinase inhibition similar to active adenomas (Fig. 4).

pS6rp Immunostaining

Of each of GHoma and NFPA, seven samples were subjected to immunostaining for pS6rp and assessed by an experienced pathologist. The level of pS6rp staining was clearly higher in GHoma group (Fig. 5 and ESM 2).

Discussion

Since mTOR is a key regulator of cell growth, mTOR inhibitors are potential therapeutics for treatment of cancer. mTOR pathway plays a major role in numerous hematological malignancies but recently it is getting more attention in regard to the management of solid tumors. This interest is supported by clinical application of mTOR inhibitors—temsirolimus and everolimus—that have been approved for the treatment of advanced renal clear cell carcinoma and pancreatic neuroendocrine tumors [28, 29].

Previous studies concerning mTOR kinase activation in pituitary adenomas focused mainly on cell line models. A work by Zatelli and colleagues on NFPA primary cultures showed that everolimus (a rapamycin derivative) decreased the activity of mTOR and led to the reduction of cell viability affecting apoptosis and proliferation [30]. Another study on NFPAs showed relative insensitivity to rapamycin treatment that was overcome with the somatostatin analog—octreotide [31]. Similar studies were conducted on GHoma-derived cell lines. Rapamycin and everolimus had antiproliferative effect on two GHoma-related rat cell lines (GH3 and MtT/S) and primary pituitary cells isolated form dispersed GHomas, where eight out of nine (88 %) cultures showed reduction in cell viability upon drug administration [32]. However, these in vitro studies have been carried out

Table 2 The frequency of pErk active adenomas presented as percentage of samples with active mTOR in the given type of adenoma and, in parenthesis, as number of active samples/number of analyzed samples. The data are non-normally distributed

Type	Erk activity frequency	Median of pErk/elF4E	25–75 percentiles of pErk/elF4E	Minimum/Maximum detected pErk/elF4E
GHoma	100 % (14/14)	0.735	0.205–1.304	0.039/2.238
NFPA	87 % (29/33)	0.598	0.236–1.097	0-2.925
ACTHoma	100 % (6/6)	1.191	0.812–1.462	0.582/10.091
All adenomas	92 % (49/53)	0.731	0.306–1.250	0/10.091
Control	100 % (5/5)	2.103	2.031–2.979	1.691/7.684

Table 3 Correlation analysis of kinase activity and tumor features (Spearman *R*). Correlations that reached statistical significance ($p<0.05$) are italicized. Due to the small number of samples, ACTHoma group was included only in the general analysis

	pErk/elF4E	pS6rp/elF4E	Volume	Largest dimension	Knosp's grading	Ki-67 %	SSTR2A	SSTR5
Correlation analysis for GHoma								
pErk/elF4E	1.00	−0.23	0.09	0.16	0.03	−0.32	*0.57*	−0.38
pS6rp/elF4E	−0.23	1.00	−0.31	−0.32	−0.33	0.32	−0.03	−0.02
Volume	0.09	−0.31	1.00	*0.97*	0.27	−0.07	−0.29	−0.14
Largest dimension	0.16	−0.32	*0.97*	1.00	0.30	−0.05	−0.17	−0.26
Knosp's grading	0.03	−0.33	0.27	0.30	1.00	*0.62*	0.35	−0.09
Ki-67 %	−0.32	0.32	−0.07	−0.05	*0.62*	1.00	0.31	0.12
SSTR2A	*0.57*	−0.03	−0.29	−0.17	0.35	0.31	1.00	−0.23
SSTR5	−0.38	−0.02	−0.14	−0.26	−0.09	0.12	−0.23	1.00
Correlation analysis for NFPA								
pErk/elF4E	1.00	0.15	−0.22	−0.21	*−0.34*	−0.32		
pS6rp/elF4E	0.15	1.00	0.11	0.12	0.03	0.08		
Volume	−0.22	0.11	1.00	*0.97*	*0.56*	−0.07		
Largest dimension	−0.21	0.12	*0.97*	1.00	*0.60*	−0.11		
Knosp's grading	*−0.34*	0.03	*0.56*	*0.60*	1.00	0.20		
Ki-67 %	−0.32	0.08	−0.07	−0.11	0.20	1.00		
Correlation analysis for all adenomas								
pErk/elF4E	1.00	−0.02	−0.27	−0.26	*−0.30*	−0.20		
pS6rp/elF4E	−0.02	1.00	−0.07	−0.06	−0.13	0.13		
Volume	−0.27	−0.07	1.00	*0.98*	*0.54*	−0.13		
Largest dimension	−0.26	−0.06	*0.98*	1.00	*0.55*	−0.16		
Knosp's grading	*−0.30*	−0.13	*0.54*	*0.55*	1.00	0.26		
Ki-67 %	−0.20	0.13	−0.13	−0.16	0.26	1.00		

in culturing conditions that may not be a reliable model for tumor in situ environment. In this study we showed that primary cell lines derived from adenomas that did not exhibit mTOR activation in fresh frozen samples, have high level of mTOR activity in vitro, in standard culturing conditions. This may suggest that data from primary cell cultures and cell lines may not be representative for the in situ adenoma environment.

Our study that bases on analysis of the adenoma samples obtained freshly from the patients demonstrates the increased activity of mTOR kinase in all adenomas; however, this activity was observed with the highest frequency in GHomas. Also, the strength of this activity was the highest in GHomas ($p=0$, 04 GHoma vs. NFPA in *U* Mann–Whitney test). This result was confirmed in immunochemistry. To the best of our knowledge, the same approach was undertaken in one study conducted by Dworakowska and colleagues. However, it revealed no differences in the phosphorylation of mTOR kinase between different types of adenomas and in comparison to healthy pituitaries obtained from autopsy [13]. This discrepancy may be due to the different methods and samples used as controls in the studies. In our study the activity was measured as the level of S6rp phosphorylation—a protein that is robustly phosphorylated by mTOR kinase and is often used as the readout of its activation. Validity of this approach is further proved by complete abolition of S6rp phosphorylation using specific inhibitor of mTOR—rapamycin—in primary cells derived from mTOR active adenomas. However, despite finding that mTOR is hyperactive in these tumors, we did not observe any correlation between the activity of mTOR and morphopathological features in PAs, such as tumor volume, largest dimension, Ki-67 index, Knosp's grading or somatostatin receptor (SSTR) expression. In the primary cell cultures, we assessed three mTOR active adenomas (GHoma, NFPA, ACTHoma) and two mTOR inactive adenomas (two NFPAs). As mentioned above, all primary cultures from adenomas showed strong S6rp phosphorylation that was abolished in response to very low doses of highly selective mTOR inhibitor. In the experiments utilizing selective inhibitors of two major upstream kinases, mTOR activity showed to be independent of Erk or Akt. The observation that these two chief activators of mTOR do not contribute to its activity in PA suggests involvement of other regulators of mTOR activity. Lack of Erk involvement in mTOR activity is further supported by the absence of correlation between Erk and S6rp phosphorylation in fresh frozen samples.

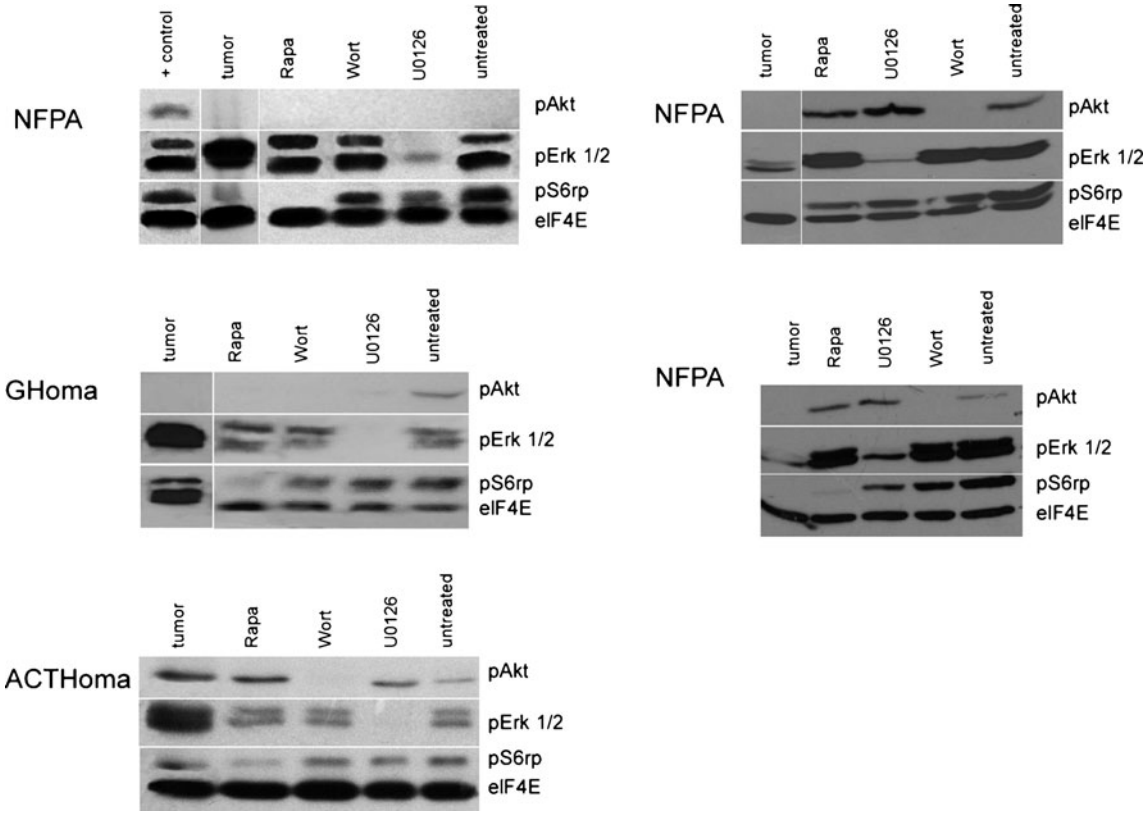

Fig. 4 Western blot from the primary cultures (48 h) on matrigel derived from adenomas with mTOR active in vivo (*left*) and mTOR non-active in vivo (*right*). All cultures had active mTOR and Erk. Akt activity was detected in three of them. All cell lines showed mTOR inhibition in response to rapamycin while inhibition of Erk and Akt did not affect mTOR activity

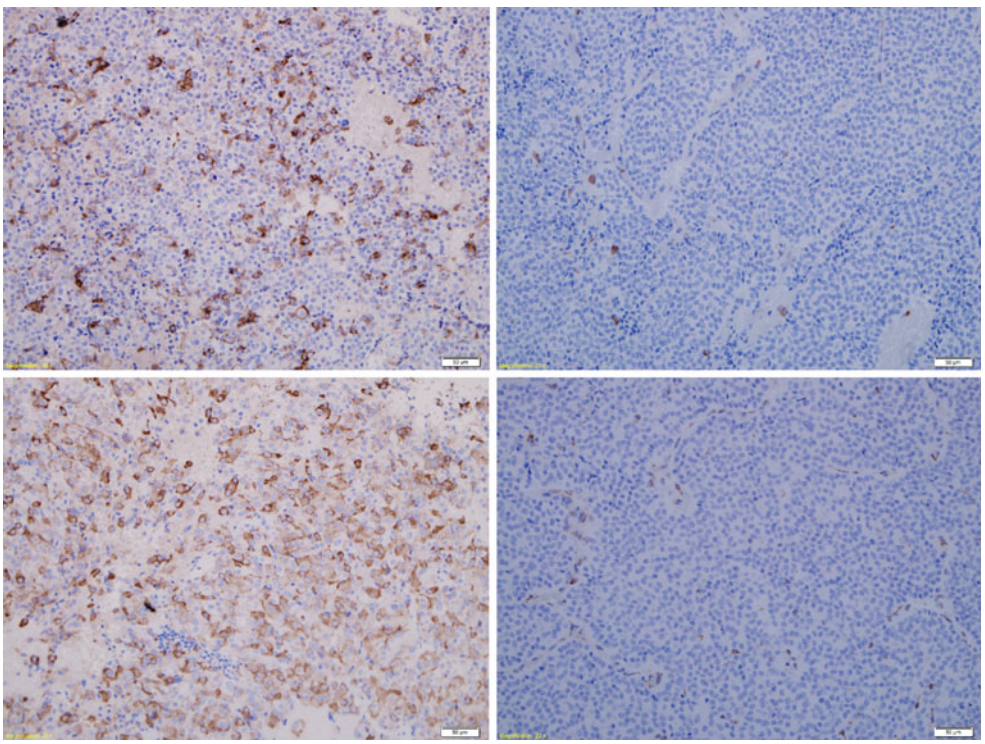

Fig. 5 Immunostaining for pS6rp as a readout of mTOR activity was performed in seven GHomas and 7 NFPAs. Representative pictures are shown. The staining is more intense in GHomas (*left*) comparing to NFPAs (*right*). Microscopic photographs for all 14 samples are provided in ESM 2. *Scale bar* = 50μm

The activity of pErk in fresh frozen samples showed a surprising pattern. It was active in all types of adenomas, but very high activity was detected in the control pituitary tissue. Our correlation analysis also supports this finding where Erk showed an inverse correlation with Knosp's grading (a marker for adenoma invasiveness). Our data are in many aspects novel and not in line with some of the previously published reports. The cause of discrepancies may be the tissue used as the pituitary control. So far most studies analyzing intracellular pathways in PAs used autopsy obtained specimen for this purpose [13–15]. However, cadaveric samples may underestimate the level of kinase activation due to the short half-life of phosphorylation [33]. In the preliminary experiments we found, that phosphoproteins of mTOR pathway are hard to detect in the pituitary autopsy samples (ESM 3). Therefore, since biopsy is not an option, it seems that fresh control tissue can be obtained only during pituitary surgery as was done in our study. Although the nature of such control tissue may be considered a limitation, since pituitary hormones in these individuals have abnormal serum levels, to our knowledge, there is no evidence for ACTH or glucocorticoids (GCs) clear effect on Erk in pituitary tissue. Furthermore, in studies performed on nonpituitary models GCs were proved to decrease Erk activity [34, 35].

Conclusion

The role of mTOR activation in PA pathogenesis still remains inconclusive. Despite some limitations of this study such as limited number of control samples, we present here, for the first time, the data indicating the high rate of mTOR activity in GHomas compared to NFPAs. Although, further studies and statistical analysis revealed lack of correlation between this activity and adenoma morphopathological features. However, as the subjects of our study were benign cases of pituitary adenomas, mTOR may still pose an interesting target for recurrent, invasive GHomas or malignant tumors. In this course, in a recent study, performed on GH3 adenoma cell line, mTOR inhibition-sensitized cell cultures to radiation suggesting possible use of such agents to overcome radioresistance [36]. Another research in this regard assessed the effect of Nelfinavir in locally invasive pituitary adenomas in vitro and in vivo [37]. This agent caused a statistically significant radiation sensitization alongside with the inhibition of S6rp phosphorylation. Hence, the role of mTOR pathway seems to be promising in radiation combined therapy of locally invasive adenomas. mTOR was also proposed as a new target for the treatment of pituitary carcinomas. However, in at least one study, a combined everolimus–octreotide therapy failed to improve the outcome of an aggressive temozolomide-resistant ACTH-secreting carcinoma [38]. Therefore, further researches elucidating the role of mTOR in pituitary neoplasms seem indispensible.

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References

1. Scheithauer BW, Gaffey TA, Lloyd RV, Sebo TJ, Kovacs KT, Horvath E, Yapicier O, Young WF, Jr., Meyer FB, Kuroki T, Riehle DL, Laws ER, Jr. (2006) Pathobiology of pituitary adenomas and carcinomas. Neurosurgery 59 (2):341–353; discussion 341–353. doi:10.1227/01.NEU.0000223437.51435.6E
2. Salehi F, Kovacs K, Scheithauer BW, Cantelmi D, Horvath E, Lloyd RV, Cusimano M (2010) Immunohistochemical expression of pituitary tumor transforming gene (PTTG) in pituitary adenomas: a correlative study of tumor subtypes. Int J Surg Pathol 18 (1):5–13. doi:10.1177/1066896909356105
3. Lloyd RJ, Kovacs K, Young WFJ, Farrell WE, Asa SL, Trouillas J, Kontogeorgos G, Sano T, Scheithauer BW, Horvath E, Watson REJ, Lindell EP, Barkan AL, Saeger W, Nose´ V, Osamura RY, Ezzat S, Yamada S, Roncaroli F, Lopes MBS, Vidal Ruibal S (2004) Tumours of the pituitary in WHO Classification of Tumours. Pathology & Genetics. Tumours of Endocrine Organs. International Agency for Research and Cancer (IARC):9–48
4. Salehi F, Agur A, Scheithauer BW, Kovacs K, Lloyd RV, Cusimano M (2009) Ki-67 in pituitary neoplasms: a review–part I. Neurosurgery 65 (3):429–437; discussion 437. doi:10.1227/01.NEU.0000349930.66434.82
5. Thapar K, Kovacs K, Scheithauer BW, Stefaneanu L, Horvath E, Pernicone PJ, Murray D, Laws ER, Jr. (1996) Proliferative activity and invasiveness among pituitary adenomas and carcinomas: an analysis using the MIB-1 antibody. Neurosurgery 38 (1):99–106; discussion 106–107
6. Ezzat S, Asa SL, Couldwell WT, Barr CE, Dodge WE, Vance ML, McCutcheon IE (2004) The prevalence of pituitary adenomas: a systematic review. Cancer 101 (3):613–619. doi:10.1002/cncr.20412
7. Beckers A, Daly AF (2007) The clinical, pathological, and genetic features of familial isolated pituitary adenomas. Eur J Endocrinol 157 (4):371–382. doi:10.1530/EJE-07-0348
8. Karhu A, Aaltonen LA (2007) Susceptibility to pituitary neoplasia related to MEN-1, CDKN1B and AIP mutations: an update. Hum Mol Genet 16 Spec No 1:R73-79. doi:10.1093/hmg/ddm036
9. Horvath A, Stratakis CA (2008) Clinical and molecular genetics of acromegaly: MEN1, Carney complex, McCune-Albright syndrome, familial acromegaly and genetic defects in sporadic tumors. Rev Endocr Metab Disord 9 (1):1–11. doi:10.1007/s11154-007-9066-9
10. Dworakowska D, Grossman AB (2009) The pathophysiology of pituitary adenomas. Best Pract Res Clin Endocrinol Metab 23 (5):525–541. doi:10.1016/j.beem.2009.05.004
11. Drakos E, Rassidakis GZ, Medeiros LJ (2008) Mammalian target of rapamycin (mTOR) pathway signalling in lymphomas. Expert Rev Mol Med 10:e4. doi:10.1017/S1462399408000586
12. Bai X, Jiang Y (2010) Key factors in mTOR regulation. Cell Mol Life Sci 67 (2):239–253. doi:10.1007/s00018-009-0163-7

13. Dworakowska D, Wlodek E, Leontiou CA, Igreja S, Cakir M, Teng M, Prodromou N, Goth MI, Grozinsky-Glasberg S, Gueorguiev M, Kola B, Korbonits M, Grossman AB (2009) Activation of RAF/MEK/ERK and PI3K/AKT/mTOR pathways in pituitary adenomas and their effects on downstream effectors. Endocr Relat Cancer 16 (4):1329–1338. doi:10.1677/ERC-09-0101
14. Musat M, Korbonits M, Kola B, Borboli N, Hanson MR, Nanzer AM, Grigson J, Jordan S, Morris DG, Gueorguiev M, Coculescu M, Basu S, Grossman AB (2005) Enhanced protein kinase B/Akt signalling in pituitary tumours. Endocr Relat Cancer 12 (2):423–433. doi:10.1677/erc.1.00949
15. Ewing I, Pedder-Smith S, Franchi G, Ruscica M, Emery M, Vax V, Garcia E, Czirjak S, Hanzely Z, Kola B, Korbonits M, Grossman AB (2007) A mutation and expression analysis of the oncogene BRAF in pituitary adenomas. Clin Endocrinol (Oxf) 66 (3):348–352. doi:10.1111/j.1365-2265.2006.02735.x
16. Noh TW, Jeong HJ, Lee MK, Kim TS, Kim SH, Lee EJ (2009) Predicting recurrence of nonfunctioning pituitary adenomas. J Clin Endocrinol Metab 94 (11):4406–4413. doi:10.1210/jc.2009-0471
17. Adjei AA, Hidalgo M (2005) Intracellular signal transduction pathway proteins as targets for cancer therapy. J Clin Oncol 23 (23):5386–5403. doi:10.1200/JCO.2005.23.648
18. Friday BB, Adjei AA (2008) Advances in targeting the Ras/Raf/MEK/Erk mitogen-activated protein kinase cascade with MEK inhibitors for cancer therapy. Clin Cancer Res 14 (2):342–346. doi:10.1158/1078-0432.CCR-07-4790
19. Roberts PJ, Der CJ (2007) Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. Oncogene 26 (22):3291–3310. doi:10.1038/sj.onc.1210422
20. Iwenofu OH, Lackman RD, Staddon AP, Goodwin DG, Haupt HM, Brooks JS (2008) Phospho-S6 ribosomal protein: a potential new predictive sarcoma marker for targeted mTOR therapy. Mod Pathol 21 (3):231–237. doi:10.1038/modpathol.3800995
21. Kim SH, Zukowski K, Novak RF (2009) Rapamycin effects on mTOR signaling in benign, premalignant and malignant human breast epithelial cells. Anticancer Res 29 (4):1143–1150. doi:29/4/1143
22. Dieterlen MT, Bittner HB, Klein S, von Salisch S, Mittag A, Tarnok A, Dhein S, Mohr FW, Barten MJ (2011) Assay validation of phosphorylated S6 ribosomal protein for a pharmacodynamic monitoring of mTOR-inhibitors in peripheral human blood. Cytometry B Clin Cytom. doi:10.1002/cyto.b.21005
23. O'Reilly KE, Warycha M, Davies MA, Rodrik V, Zhou XK, Yee H, Polsky D, Pavlick AC, Rosen N, Bhardwaj N, Mills G, Osman I (2009) Phosphorylated 4E-BP1 is associated with poor survival in melanoma. Clin Cancer Res 15 (8):2872–2878. doi:10.1158/1078-0432.CCR-08-2336
24. Ronellenfitsch MW, Brucker DP, Burger MC, Wolking S, Tritschler F, Rieger J, Wick W, Weller M, Steinbach JP (2009) Antagonism of the mammalian target of rapamycin selectively mediates metabolic effects of epidermal growth factor receptor inhibition and protects human malignant glioma cells from hypoxia-induced cell death. Brain 132 (Pt 6):1509–1522. doi:10.1093/brain/awp093
25. Abramoff MD, Magalhaes PJ, Ram SJ (2004) Image processing with ImageJ. Biophotonics Int 11(7): 36–42
26. Wlodarski P, Kasprzycka M, Liu X, Marzec M, Robertson ES, Slupianek A, Wasik MA (2005) Activation of mammalian target of rapamycin in transformed B lymphocytes is nutrient dependent but independent of Akt, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase, insulin growth factor-I, and serum. Cancer Res 65 (17):7800–7808. doi:10.1158/0008-5472.CAN-04-4180
27. Knosp E, Steiner E, Kitz K, Matula C (1993) Pituitary adenomas with invasion of the cavernous sinus space: a magnetic resonance imaging classification compared with surgical findings. Neurosurgery 33 (4):610–617; discussion 617–618
28. Strimpakos AS, Karapanagiotou EM, Saif MW, Syrigos KN (2009) The role of mTOR in the management of solid tumors: an overview. Cancer Treat Rev 35 (2):148–159. doi:10.1016/j.ctrv.2008.09.006
29. Yao JC, Shah MH, Ito T, Bohas CL, Wolin EM, Van Cutsem E, Hobday TJ, Okusaka T, Capdevila J, de Vries EG, Tomassetti P, Pavel ME, Hoosen S, Haas T, Lincy J, Lebwohl D, Oberg K (2011) Everolimus for advanced pancreatic neuroendocrine tumors. N Engl J Med 364 (6):514–523. doi:10.1056/NEJMoa1009290
30. Zatelli MC, Minoia M, Filieri C, Tagliati F, Buratto M, Ambrosio MR, Lapparelli M, Scanarini M, Degli Uberti EC (2010) Effect of everolimus on cell viability in nonfunctioning pituitary adenomas. J Clin Endocrinol Metab 95 (2):968–976. doi:10.1210/jc.2009-1641
31. Cerovac V, Monteserin-Garcia J, Rubinfeld H, Buchfelder M, Losa M, Florio T, Paez-Pereda M, Stalla GK, Theodoropoulou M (2010) The somatostatin analogue octreotide confers sensitivity to rapamycin treatment on pituitary tumor cells. Cancer Res 70 (2):666–674. doi:10.1158/0008-5472.CAN-09-2951
32. Gorshtein A, Rubinfeld H, Kendler E, Theodoropoulou M, Cerovac V, Stalla GK, Cohen ZR, Hadani M, Shimon I (2009) Mammalian target of rapamycin inhibitors rapamycin and RAD001 (everolimus) induce anti-proliferative effects in GH-secreting pituitary tumor cells in vitro. Endocr Relat Cancer 16 (3):1017–1027. doi:10.1677/ERC-08-0269
33. Murphy LO, Smith S, Chen RH, Fingar DC, Blenis J (2002) Molecular interpretation of ERK signal duration by immediate early gene products. Nat Cell Biol 4 (8):556–564. doi:10.1038/ncb822 ncb822
34. Kassel O, Sancono A, Kratzschmar J, Kreft B, Stassen M, Cato AC (2001) Glucocorticoids inhibit MAP kinase via increased expression and decreased degradation of MKP-1. EMBO J 20 (24):7108–7116. doi:10.1093/emboj/20.24.7108
35. Kumamaru E, Numakawa T, Adachi N, Kunugi H (2011) Glucocorticoid suppresses BDNF-stimulated MAPK/ERK pathway via inhibiting interaction of Shp2 with TrkB. FEBS Lett 585 (20):3224–3228. doi:10.1016/j.febslet.2011.09.010
36. Sukumari-Ramesh S, Singh N, Dhandapani KM, Vender JR (2011) mTOR inhibition reduces cellular proliferation and sensitizes pituitary adenoma cells to ionizing radiation. Surg Neurol Int 2:22. doi:10.4103/2152-7806.77029
37. Zeng J, See AP, Aziz K, Thiyagarajan S, Salih T, Gajula RP, Armour M, Phallen J, Terezakis S, Kleinberg L, Redmond K, Hales RK, Salvatori R, Quinones-Hinojosa A, Tran PT, Lim M (2011) Nelfinavir induces radiation sensitization in pituitary adenoma cells. Cancer Biol Ther 12 (7):657–663. doi:17172
38. Jouanneau E, Wierinckx A, Ducray F, Favrel V, Borson-Chazot F, Honnorat J, Trouillas J, Raverot G (2012) New targeted therapies in pituitary carcinoma resistant to temozolomide. Pituitary 15 (1):37–43. doi:10.1007/s11102-011-0341-0