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Immunohistochemical Analysis of mTOR Pathway Expression in Gastric Neuroendocrine Tumors

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Abstract

Background: The mammalian target of rapamycin (mTOR) is an important regulator of cell proliferation and protein translation and is activated in various malignancies. Expression of mTOR cascade components in gastric neuroendocrine tumors (NETs), however, has not yet been fully explored.

Aims: The goal of the present study was to assess the activation of mTOR and its upstream and downstream components in gastric NETs using immunohistochemistry and to investigate the relationship between expression and clinicopathological data.

Methods: The expression of phosphorylated mTOR (p-mTOR) and its major target the eukaryotic initiation factor 4E-binding protein 1 (p4EBP1), phospho-phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (pPIK3CA), phospho-protein kinase B (pAkt), phospho-phosphatase and tensin homolog (pPTEN), and phospho-tuberous sclerosis 2 (pTSC2) were examined in a series of 35 gastric NETs.

Results: All samples from the 35 patients showed expression of the PI3K catalytic subunit PIK3CA and the mTOR inhibitor TSC2. The p-mTOR was expressed in 88.57%, pPTEN in 97.14%, and pAkt in 65.7% of the examined tumors. The mTOR effector p4E-BP1 was expressed in 88.57% of cases. In addition, the p-mTOR positive rate correlated with Ki-67 expression. In fact, patients with Ki-67 ≤ 2 had higher p-mTOR positive rates (p=0.032); however, no significant correlations between p-mTOR positivity and selected clinicopathological characteristics were observed.

Conclusions: In conclusion, these data demonstrate high mTOR activation in gastric NETs, suggesting that mTOR pathway inhibition may be a possible therapeutic strategy for treatment of gastric NETs.

Keywords: mTOR pathway; Neuroendocrine tumor; Molecular therapy

Abbreviations

4EBP1-eukaryotic initiation factor 4E-binding protein 1; Akt-Protein kinase B; ENETS-European Neuroendocrine Tumor Society; MEN 1 (or 2) multiple neuroendocrine neoplasia type 1 (or 2); mTOR - mammalian target of rapamycin; NEC– neuroendocrine carcinomas; NET- neuroendocrine tumor; PDK-1- 3-phosphoinositide-dependent protein kinase; PIK3CA- phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; PTEN- phosphatase and tensin homolog; RBS6K- ribosomal protein S6 kinase; TSC2- tuberous sclerosis 2; WHO- World Health Organization.

Introduction

The incidence of gastric neuroendocrine tumors (NET) has increased rapidly over the last decade, likely due to the rising number of gastroscopies [1]. Four different biologically relevant types of gastric NET have been described [2,3]. Type 1 comprises about 70-80% of gastric NETs and is associated with atrophic gastritis. Patients show multiple small tumors in the mucosa. Type 2 is rare and is associated with a duodenal gastrinoma in patients with the diagnosis of multiple neuroendocrine neoplasia type 1 (MEN 1) syndrome. Therefore, the gastric mucosa in these patients does not exhibit atrophy but displays hyperplasia of chief cells and parietal cells. Type 3 tumors are sporadic gastric NETs that are mostly larger single tumors at the time of diagnosis with an increased risk of lymph node and liver metastasis [4]. Poorly differentiated neuroendocrine carcinomas (NEC) of the stomach are considered to be a forth type of NET, although these tumors have not been included in the original classification by Rindi [5]. Gastric NET and NEC are currently treated with multidisciplinary approaches, including a combination of surgery, chemotherapy, and targeted molecular therapy [5,6].

The mTOR inhibitor everolimus and the multikinase inhibitor sunitinib have previously gained approval from the FDA for the treatment of unresectable, locally advanced or metastatic pancreatic NET; however, little is known about the mTOR pathway in gastric NETs. In this respect, a detailed protein expression map of mTOR-pathway components in gastric NETs is a good starting point to define

specific expression patterns that are predictive of clinical response, as suggested for other malignancies [7]. Additionally, this approach could facilitate investigation of the prognostic implications of these molecules.

The mTOR protein is a Ser/Thr protein kinase that is formed by two different protein complexes, mTORC1 and mTORC2. This complex is activated via phosphorylation by an upstream signaling cascade. Activation of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) via different receptors results in phosphorylation of 3-phosphoinositide-dependent protein kinase-1 (PDK-1) at Tyr 373/376 and Ser24. Then, pPDK1 phosphorylates Akt, and pAKT phosphorylates mTOR, while the tumor suppressor proteins tuberous sclerosis (TSC1), TSC2, and phosphatase and tensin homolog (PTEN) inhibit phosphorylation of mTOR. The known downstream targets of p-mTOR are the eukaryotic translation initiation factor 4E-binding protein (4EBP1) and the ribosomal protein S6 kinase (RBS6K), which mediate an increase in protein synthesis and cell growth [8,9].

In the present study, we used immunohistochemistry to investigate the expression of p-mTOR, its downstream target p4EBP1, the upstream regulators pAkt and pPIK3CA, and also the suppressor proteins pTSC2 and pPTEN. We examined these expression data in the context of clinicopathological variables.

Patients, Materials, and Methods

Patient Characteristics

A total of 35 patients who received treatment for gastric NETs at the Charite University Hospital were included in this study. Of these, 19 were female (54.3%), and the median age for all 35 patients was 48.5 years. In 24 patients, NETs were associated with chronic atrophic gastritis (type 1 NET). Three of these patients had a known MEN1 syndrome (type 2 NET), and four patients had sporadic gastric NET (type 3 NET). Another four patients with the diagnosis of gastric NEC (type 4 tumors) were included. All tumors were graded and staged according to the latest WHO (WHO 2010) and ENETS classification (Table 1).

Case	Age (years)/Sex	Grading	Type
1	50/F	G1	2
2	50/F	G1	1
3	47/F	G2	3
4	54/M	G1	1
5	59/M	G1	1
6	69/M	G3	4
7	74/M	G1	2
8	57/M	G1	1
9	72/F	G2	1
10	65/F	G1	1
11	57/M	G1	1
12	46/F	G1	1
13	57/M	G3	4
14	60/F	G1	1
15	57/M	G2	3
16	56/F	G1	1
17	73/M	G2	3
18	60/M	G1	1
19	75/F	G3	4
20	63/F	G3	1
21	54/F	G2	1
22	55/F	G1	1
23	49/F	G1	1
24	46/M	G1	1
25	73/M	G2	3
26	68/F	G1	1
27	66/M	G2	4
28	80/M	G1	1
29	52/F	G1	1
30	56/F	G1	1
31	73/M	G1	1
32	53/F	G1	1
33	43/F	G2	1
34	72/M	G1	1
35	84/F	G1	2

Table 1: Clinicopathological characteristics of the gastric NET patients. M-male, F-female, G1- well differentiated neuroendocrine tumor, G2-moderately differentiated neuroendocrine tumor, G3- poorly differentiated neuroendocrine tumor

Construction of Tissue Microarrays

For immunohistochemical analysis of mTOR pathway expression, tissue microarrays were constructed using a semiautomatic tissue arrayer (TMA Grandmaster, 3DHistech, Budapest, Hungary). A representative tumor-bearing paraffin block of formalin-fixed tissue was selected for each case. Hematoxylin and eosin-stained slides were prepared from the tissue blocks, and tumor-bearing areas were marked on the slides. Both the selection of blocks and the marking of tumor-bearing areas were conducted by a pathologist specialized in NET pathology (RA). Subsequently, one or two tissue cores with a diameter of 1 mm were punched from the tumor-bearing areas and transferred to a donor paraffin block.

Immunohistochemistry

Tissue microarray sections (2-3 um thick) were cut and incubated with antibodies directed against p-mTOR (Ser2448) (clone 49F9, 1:100; Cell Signaling Technology, Danvers, MA, USA), pTSC2 (polyclonal, 1:100; Cell Signaling Technology), pAkt (polyclonal,

1:200; Abcam, Cambridge, MA, USA), p4EBP1 (clone 235B4, 1:100; Cell Signaling Technology), pPTEN (monoclonal, 1:100; Cell Signaling Technology), and pPIK3CA (1:100; Cell Signaling Technology). Omission of the primary antibody served as a negative control. Ki-67 staining (clone M7240, 1:100; Dako, Glostrup, Denmark) was accomplished on whole slides. Staining was performed in a Benchmark XT autostainer (Ventana, Tuscon, AZ, USA), according to the manufacturer's protocol. Samples were considered positive when at least 50% of tumor cells showed a moderate or strong expression of the detected protein.

Statistical analysis

Statistical analyses were conducted using SPSS 20 statistical software (SPSS Inc., Chicago, IL, USA). The associations between immunohistochemical marker expression and clinicopathological variables were determined by a chi-squared test. The correlation of the mTOR pathway components expression scores with each other and with proliferation indices was examined by Spearman's rank order correlation. All tests were two-tailed, and the results were considered significant when $p<0.05$. In addition, survival analysis was performed using the Kaplan-Meier log-rank test.

Results

Expression of mTOR pathway components in gastric NET

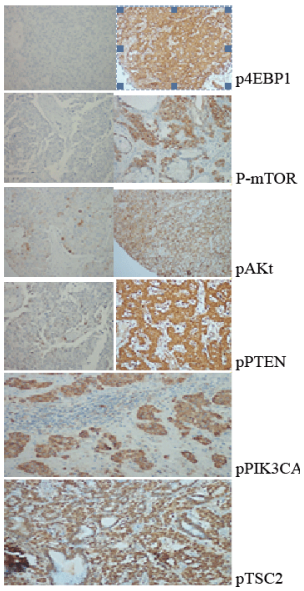

Figure 1: Representative immunohistochemical stainings for the expression of phosphorylated 4E-BP1 binding protein (p4E-BP1), phosphorylated mammalian target of rapamycin (p-mTOR), pAKt, pPTEN, pPIK3CA and pTSC2 in gastric neuroendocrine tumors (original magnification, 200X), Left-negative staining, right-positive staining. For pPIK3CA and pTSC2 only positive staining is shown.

We analyzed the expression of six proteins that are known to play important roles in the mTOR pathway. Samples from all 35 patients exhibited expression of the pPIK3CA and pTSC2.

Case	p-mTOR	p-Akt	pBP1	pPTEN
1	+	+	+	+
2	+	+	+	+
3	+	+	+	+
4	+	+	+	+
5	+	+	-	+
6	+	-	+	+
7	+	-	+	+
8	+	-	+	+
9	-	-	+	+
10	+	-	+	+
11	+	-	+	+
12	+	+	+	+
13	-	-	-	-
14	+	-	+	+
15	-	+	-	+
16	+	+	+	+
17	+	-	+	+
18	+	+	+	+
19	+	+	+	+
20	+	+	+	+
21	+	-	+	+
22	+	+	+	+
23	+	+	+	+
24	+	+	+	+
25	-	+	-	+
26	+	+	+	+
27	+	-	+	+
28	+	+	+	+
29	+	+	+	+
30	+	-	+	+
31	+	+	+	+
32	+	+	+	+
33	+	+	+	+
34	+	+	+	+
35	+	+	+	+

Table 2: Immunohistochemical results of gastric NET

In addition, p-mTOR was expressed in 88.57%, pPTEN in 97.14%, and pAKT in 65.7% of the examined tumors. The mTOR effector p4EBP1 was expressed in 88.57% of cases (Table 2 and Figure 1).

Correlation between mTOR pathway components in gastric NETs

We next examined the correlation between the mTOR pathway components. As expected there was a positive, albeit weak, linear correlation between the expression of pAkt and p-mTOR (p=0.496) as well as a strong linear correlation between the expression p-mTOR and the effector protein p4E-BP1 (p=0.001). We could not, however, test the associations between expression of pPTEN and pPIK3CA or expression of pTSC2 and p-mTOR, because all samples were positive for pPIK3CA and pTSC2.

Correlation of mTOR pathway components with tumor type, grading, tumor stage, and metastasis

No statistically significant differences were detected in the p-mTOR expression between the Type 1, Type 2, Type 3, and Type 4 NETs (p=0.082). In addition, no statistically significant differences in p-mTOR expression in metastatic versus non-metastatic tumors were found (p=0.238). The rate of p-mTOR positivity correlated with Ki-67 staining, and more specifically, the patients with Ki-67≤2 had higher p-mTOR positive rates (p=0.032; Tables 2 and 3). No statistically significant differences were detected with respect to p-mTOR expression and ENETS stage classification (p=0.119).

Furthermore, no statistically significant differences in pPTEN expression were detected with respect to tumor type (p=0.092) or metastasis status (p=0.085), but a statistically significant difference was found in pPTEN expression with regard to Ki-67 level, higher positivity by lower Ki-67 levels (p=0.004). No statistically significant differences in pPTEN expression levels in association with ENETS stage classification (p=0.186). In an analysis of pAkt expression, we failed to find any statistically significant differences for any analyzed parameter (Table 3).

	n	p-mTOr		p-Akt		p-4BEP1		p-PTEN	
		positive	p	positive	p	positive	p	positive	p
Tumor typ									
Typ1	24	22 (91.7)	0.082	17 (70.8)	0.438	24 (100)	0.001	24 (100)	0.092
Typ2	3	3 (100)		2 (66.7)		3 (100)		3 (100)	
Typ3	4	2 (50)		3 (75)		2 (50)		2 (50)	
Typ4	4	3 (75)		1 (25)		3 (75)		3 (75)	
Ki67									
<=2	21	21 (100)	0.032	15 (71.4)	0.423	20 (95.2)	0.242	21(100)	0.004
2-20	11	8 (72.7)		7 (63.6)		9 (81.8)		11(100)	
>=20	3	2 (66.7)		1 (33.3)		2 (66.7)		2 (66.7)	
Metastasis									
yes	9	7 (77.7)	0.238	4 (44.4)	0.119	6 (66.7)	0.017	8 (88.9)	0.085
no	26	24 (92.3)		19 (73.1)		25(96.1)		26(100)	
ENETS									
Stage 0	15	14 (93.3)	0.119	12 (78.5)	0.222	15(100)	0.002	15(100)	0.186
Stage 1	10	10 (100)		5 (50)		10(100)		10(100)	
Stage 2	3	2 (66.6)		3 (100)		2 (66.7)		3 (100)	
Stage 3	2	2 (100)		1 (50)		2 (100)		2 (100)	
Stage 4	5	3 (60)		2 (40)		2 (40)		4 (80)	

Table 3: Relation between the expression of p-mTOR, pAkt, p4BEP1, pPTEN and clinico-pathological characteristics

Higher p4EBP1 expression was found in Type 1 and Type 2 NETs compared to Type 3 and Type 4 NETs (p= 0.001). This expression was also higher in non-metastatic tumors than in metastatic tumors (p=0.017). We also detected statistically significant differences with respect to ENETS stage classification, with the lower stages exhibiting higher positivity (p=0.002). In contrast to other described markers, no statistically significant differences in expression of p4EBP1 were found with respect to Ki-67 levels (p=0.242). Both pTSC2 and pPIK3CA were

positive in all cases. Additionally, we did not detect statistically significant differences in mTOR pathway expression with respect to survival.

Discussion

Expression of several mTOR pathway components has previously been assessed in pancreatic NET [10-14], but the functional activation of the PI3K/AKT/mTOR signaling pathway has never been extensively investigated in gastric NETs. Due to the development of selective inhibitors, clinical interest in the mTOR pathway has increased in recent years, and several preclinical trials have been conducted to test the efficacy of these inhibitors in different human malignancies, including NETs [15-17].

In this study, we report differential expression of pPIK3CA, pPTEN, pAkt, pTSC2, p-mTOR, and p4EBP1 in a cohort of gastric NETs. Compared with previous studies, our study included the largest number of patients for the investigation of the mTOR signaling pathway of gastric NETs. The immunohistochemistry analyses of gastric NETs identified the expression of p-mTOR in 88.57%, pPTEN in 97.14%, and pAkt in 65.7% of the examined tumors. The mTOR effector p4EBP1 was expressed in 88.57% of cases, while pTSC2 and pPIK3CA were expressed in all cases. The expression of p-mTOR was different with respect to Ki-67 status and p4EBP1 expression according to tumor type, metastasis status, and ENETS stage classification. Activation of the mTOR pathway would be expected to be associated with more aggressive clinical behavior. The proliferative marker Ki-67 has been widely used as a prognostic indicator for NETs. In contrary with observations in a previous study of gastroenteropancreatic NET [12], we did not find that expression of p-mTOR was associated with higher proliferative index in gastric NETs.

All samples were examined by immunohistochemistry, as this is the main technique used to assess protein expression or phosphorylation in paraffin-embedded tissues. To comprehensively assess mTOR signaling, future studies should expand on immunohistochemistry to include kinase assays, signaling blots, and transcriptional and translational assays to comprehensively assess mTOR signaling. Our study is important because mTOR pathway activation has not been adequately explored in gastric NETs. Thus, we provide compelling rationale for future studies of mTOR activation and mTOR inhibition as a therapeutic strategy in patients with gastric NETs.

In the present study, not all cases expressing p-mTOR co-expressed pAkt. This result suggests that the activation of mTOR in gastric NETs is regulated not only by the PI3K-Akt pathway but may also be transmitted through PDK1 without activation of Akt, as previously described in a study by Tan at al. [18]. Vasudevan at al. [19] found that PIK3CA may promote cancer through both Akt-dependent and Akt-independent mechanisms.

Previous reports on the prognostic role of mTOR pathway activation in human tumors have presented controversial findings. In some studies, activation of the mTOR pathway was found to be a favorable factor, such as for ovarian cancer [20], while others reported contradictory results in renal, biliary tract and breast carcinoma [21-24]. Discrepant results may be due to differences in various factors related to study design. For example, sample size, non-uniform treatment, insufficient follow up, or different thresholds for positivity may vary among studies, as these factors were determined by the authors of each report. Here, we also did not detect any statistically significant differences in mTOR pathway expression with respect to survival. The most likely reason for this finding was the small number of cases. A small sample size reduces the probability of achieving statistically significant results. However, our results provide the rationale for performing additional studies on mTOR activation in a larger population of patients.

In summary, despite differences in expression and correlations between expression of different components and clinicopathological data, our results demonstrate that the components of the mTOR pathway are highly expressed in gastric NETs and may, therefore, provide a potential therapeutic target for treatment of these tumors. Although the sample size in this study was limited, this is the first study to determine the expression of activated mTOR in gastric NETs. A high expression rate of p-mTOR pathway components in gastric NETs suggests that the recently described mTOR inhibitors may be effective therapeutic agents for treatment of this group of tumors. Additional research, including larger numbers of patients and clinical trials to investigate the mTOR inhibitors, should be considered in the future.

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