endocrine abnormalities prior to surgery and all tumors were diagnosed as non-functional GI NET. The specimens had all been fixed in 10% formalin and embedded in paraffin. The clinicopathological features are summarized in Table 1. We classified the status of age (years) into <60 or ≥60 based on the reported average year of NET patients in Japan.[2] Epidemiological studies in Japanese patients of GI NET demonstrated that the frequency of *MEN1* genetic abnormalities was only 1%.[2,16] However, further investigation is required for clarification. Research protocols for this study were approved by the Ethics Committee at the Tohoku University School of Medicine (2008-122) and Sendai Red Cross Hospital (No. 32).

Immunohistochemistry. Tissue specimens were immunostained using a biotin-streptavidin method with a Histofine kit (Nichirei Co. Ltd, Tokyo, Japan: phospho-ribosomal protein [p-S6], phospho-eukaryotic initiation factor 4-binding protein 1 [p-4EBP1], p-ERK, sstr subtypes and Ki-67) and an EnVision method (Dako, Kyoto, Japan: p-mTOR and p-IGF-1R). In the present study, we did not evaluate the immunoreactivity of sstr4, because sstr4 was rarely reported in NET cells.[17,18] The characteristics of antibodies used in our immunostaining are summarized in Table 2. Antigen retrieval for p-mTOR, p-S6, p-4EBP1, and p-ERK analyses was performed by heating the slides in a microwave at 500 W for 15 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0). Antigen retrieval for Ki-67 and sstrs was performed by heating the slides in an autoclave at 121°C for 5 min in citrate acid buffer. No treatment for antigen retrieval was performed in immunostaining for p-IGF-1R. These slides were further incubated with primary antibodies for 36–48 h in a moist chamber at 4°C. The antigen-antibody complex was then visualized with 3,3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris-HCl buffer, pH 7.6 and 0.006% H_2O_2) and counterstained with hematoxylin. Immunoreactivity for p-mTOR, p-4EBP1, p-S6 and p-ERK was detected in the cytoplasm of tumor cells, and classified into three groups as follows: score 0, negative; score 1, weakly positive; score 2, strongly positive (Table 3). Immunoreactivity for sstr subtypes was detected in the membrane or cytoplasm of tumor cells. An evaluation of immunoreactivity of sstrs was performed as previously reported (Table 3).[5]

Table 1. Summary of clinicopathological findings in 52 non-functioning neuroendocrine tumor cases examined in the present study

	No. cases (n = 52)
Age (years)	
<60	35 (67.3%)
≥60	17 (32.7%)
Mean ± SD	51.3 ± 15.4
Gender	
Male	32 (61.5%)
Female	20 (38.5%)
Localization†	
Foregut	15 (28.9%)
Midgut	2 (3.8%)
Hindgut	35 (67.3%)
Lymph metastasis	
Presence	3 (5.8%)
Absence	49 (94.2%)
Vascular invasion	
Presence	14 (26.9%)
Absence	38 (73.1%)

†Details of localization are as follows: foregut, 15 (lung, 5; stomach, 4; bronchus, 2; duodenum, 2; liver, 1; middle ear, 1); midgut, 2 (appendix, 2); hindgut 36 (rectum, 35; sigmoid colon, 1).

Immunoreactivity for p-IGF-1R was classified into two groups as follows: score 0, negative; score 1, positive. Representative illustration of immunohistochemistry is shown in Figure 1. Ki-67 immunoreactivity was evaluated in more than 1000 cells and the percentage of immunoreactivity (i.e. labeling index [LI]) was subsequently obtained. We scored the Ki-67 LI followed by the histopathological grade in NET recently defined by the European Neuroendocrine Tumor Society (ENETS) as follows: score 0, <2%; score 1, 2–20%; score 2, >20% (Table 3).[19] Two of the authors (S.I. and Y.M.) independently evaluated immunoreactivity and the cases of interobserver differences of more than 5% were re-evaluated together using double-headed light microscopy. Intraobserver differences were <5%.

Cell culture and reagents. NCI-H727 (H727), a human bronchial NET cell line, was purchased from the American Type Culture Collection (Manassas, VA, USA). COLO320-DM (COLO), a human colon NET cell line, was purchased from The Health Science Research Resources Bank (Osaka, Japan). These cell lines were maintained in RPMI-1640 medium (Sigma Aldrich Co., St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Nichirei Co. Ltd, Tokyo, Japan). Cells were maintained at 37°C, 95% relative humidity and 5% CO_2. Rapamycin was purchased from Wako (Osaka, Japan).

Cell proliferation assay. The status of cell proliferation of H727 and COLO cells was determined using WST-8 (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2, 4-disulfophenyl]-2H-tetrazolium monosodium salt) method (Cell Counting kit-8; Dojindo Inc., Kumamoto, Japan). Cells were seeded in 96-well plates at a density of 5000 cells/well. After 24 h of incubation, different concentrations of rapamycin were added into the medium. Compound was added with the exchange of medium every 3 days and measured for 3, 6 and 9 days (H727) or 1, 2 and 3 days (COLO). The medium including reagents was changed every 3 days. A volume of 10 μL of 5 mM WST-8 was added and the plates were then incubated for 1–4 h at 37°C in 95% relative humidity and 5% CO_2. The resulting optical densities (OD; 450 nm) were obtained using a Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The status of cell proliferation (%) was calculated according to the following equation: (cell OD value after treatment with test materials/vehicle control cell OD value) ×100.

Statistical analysis. We used hierarchical clustering analysis to sort the results of the immunohistochemistry and to further evaluate the correlation of immunohistochemical data with clinicopathological findings of individual NET cases. Hierarchical clustering analysis attempts to identify homogeneous subgroups of the cases examined are as reported by Eisen *et al.*[20] The correlation between individual cases and cell signaling factors is depicted graphically as a dendrogram in which branch length is determined by the distance between the results of the immunohistochemistry. Data were subjected to hierarchical clustering analysis and visualization using Cluster and TreeView, respectively (downloaded from the Eisen Lab, Barkley, CA, USA). Chi-squared tests were used to determine which markers contributed to the formation of individual clusters. The statistical analysis on the results of cell proliferation was analyzed with Sheffe test (StatView ver. 5.0, SAS institute, Cary, NC, USA). A P-value < 0.05 indicated the statistical significance in this study.

Results

Immunoreactivity in cell-signaling molecules in NET cases. Results of immunohistochemical staining of sstr subtypes, activated (phosphorylated) forms of intracellular signaling factors (mTOR, 4EBP1, S6 and ERK) and IGF-1R examined are summarized in Table 4 and Figures 2 and 3. Immunoreactivity for

Table 2. Antibodies and their conditions of immunostaining

Primary antibody	Dilution	Source	Antigen retrieval	Positive control
p-mTOR	1/50	Cell Signaling Technology	MW	Rectum
p-4EBP1	1/50	(Beverly, MA, USA)		
p-S6	1/100			
p-p44/42 MAPK (p-ERK)	1/100			
sstr1, 2A, 2B, 3, 5	1/1000	Gramsh Laboratories (Schwabhausen, Germany)	MW	Pancreas
p-IGF-1R	1/100	Abnova (Taipei, Taiwan)	No treatment	Breast carcinoma
Ki-67	1/100	Dako Cytomation (Glostrup, Denmark)	AC	Tonsil

AC, autoclave treatment; 4EBP1, eukaryotic initiation factor 4-binding protein 1; IGF-1R, insulin-like growth factor 1 receptor; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; MW, microwave treatment; S6, ribosomal protein s6; sstr, somatostatin receptor.

Table 3. Summary of scoring of immunoreactivity used in the present study

Primary antibody	Score 0	Score 1	Score 2
p-mTOR	Negative	Weakly	Strongly
p-4EBP1		positive	positive
p-S6			
p-ERK			
Ki-67 LI	<2%	2–20%	>20%
sstr1, 2A, 2B, 3, 5	Negative	Positive	
p-IGF-1R			

4EBP1, eukaryotic initiation factor 4-binding protein 1; IGF-1R, insulin-like growth factor 1 receptor; LI, labeling index; mTOR, mammalian target of rapamycin; S6, ribosomal protein s6; sstr, somatostatin receptor.

p-mTOR, p-4EBP1, p-S6 and p-ERK was detected in the cytoplasm of tumor cells in 33 (63.5%), 43 (82.7%), 27 (51.9%) and 18 (34.6%) of 52 NET cases examined, respectively. Immunoreactivity for sstr1, 2A, 2B, 3, 5 and p-IGF-1R was detected in the membrane or cytoplasm of tumor cells in 27 (51.9%), 48 (92.3%), 20 (38.5%), 29 (55.8%), 39 (75.0%) and 38 (73.1%) of 52 cases examined, respectively.

Hierarchical clustering analysis of immunohistochemical results in individual clusters. Hierarchical clustering analysis was applied to results of the immunohistochemistry in NET cases and the correlation was subsequently displayed graphically using the computer program, Cluster and TreeView (downloaded from Eisen Lab; Fig. 4). The patterns of each sstr subtypes obtained were nearly identical in terms of staining patterns in the great majority of tumors, that is, co-expressing all or none of these markers, especially in those of sstr2A and 5. In addition, there was an almost identical scoring pattern among p-IGF-1R, p-mTOR and p-4EBP1 and also between p-ERK and p-S6, respectively, which indicated that activation of S6 was correlated more with the MEK/ERK pathway rather than the PI3K/Akt pathway in GI NET cases.

The results showed that the 52 NET cases examined were basically classified into two clusters, Cluster I (18 cases) and Cluster II (34 cases), and Cluster II was further sub-classified into Cluster IIa (15 cases) and Cluster IIb (17 cases) according to the branch length, which represents the correlation of the scoring data (Fig. 4). Two cases belonging to Cluster II were eliminated because of the branch length.

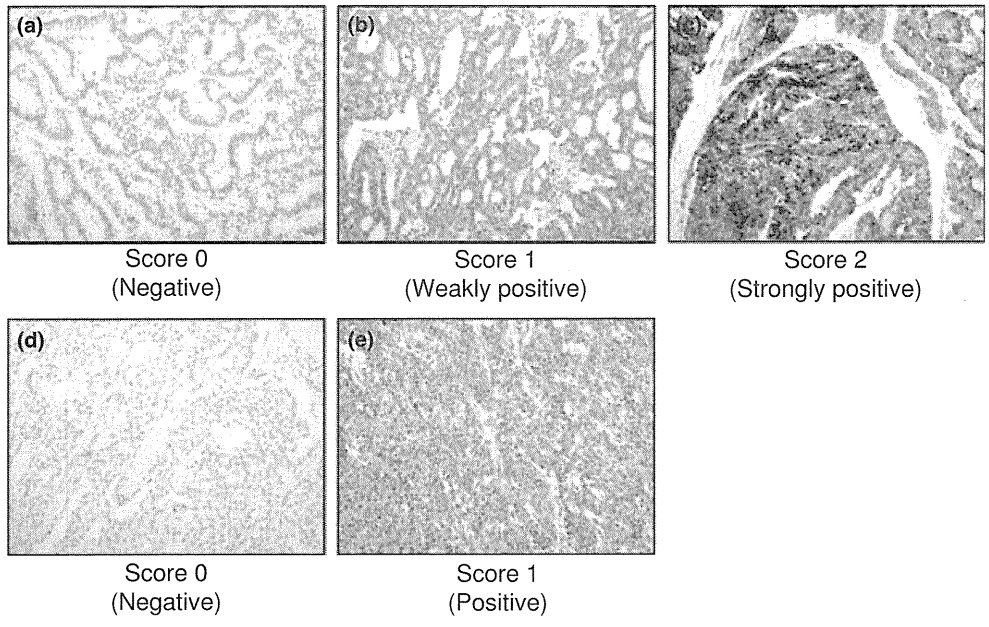

Fig. 1. Representative illustrations of immunohistochemistry. Results of immunohistochemistry were evaluated according to Table 3. (a) p-4EBP1 (score 0, negative); (b) p-4EBP1 (score 1, weakly positive); (c) p-4EBP1 (score 2, strongly positive); (d) somatostatin receptor 1 (sstr1) (score 0, negative); (e) sstr1 (score 1, positive). (a–e) Original magnification, ×100.

doi: 10.1111/j.1349-7006.2010.01657.x

Table 4. Summary of scoring of immunohistochemistry in the present study of neuroendocrine tumor cases

Total (n = 52)	Score 0	Score 1	Score 2	Total (n = 52)	Score 0	Score 1
p-mTOR	19 (36.5%)	29 (55.8%)	4 (7.7%)	sstr1	25 (48.1%)	27 (51.9%)
p-4EBP1	9 (17.3%)	33 (63.5%)	10 (19.2%)	sstr2A	4 (7.7%)	48 (92.3%)
p-S6	25 (48.1%)	24 (46.2%)	3 (5.8%)	sstr2B	32 (61.5%)	20 (38.5%)
p-ERK	34 (65.4%)	18 (34.6%)	0 (0.0%)	sstr3	23 (44.2%)	29 (55.8%)
Ki-67	39 (75.0%)	12 (23.1%)	1 (1.9%)	sstr5	13 (25.0%)	39 (75.0%)
				p-IGF-1R	14 (26.9%)	38 (73.1%)

4EBP1, eukaryotic initiation factor 4-binding protein 1; IGF-1R, insulin-like growth factor 1 receptor; mTOR, mammalian target of rapamycin; S6, ribosomal protein s6; sstr, somatostatin receptor.

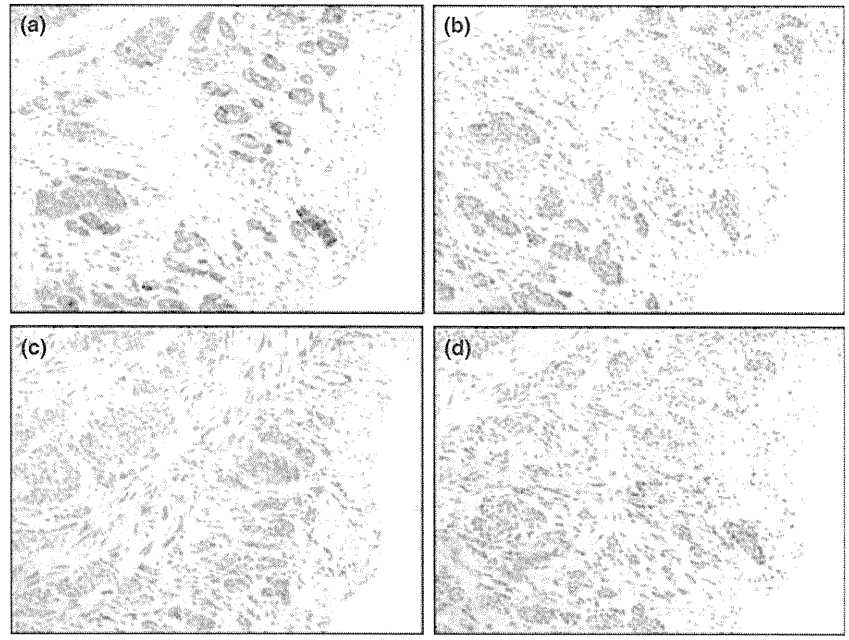

Fig. 2. Representative illustrations of immunohistochemistry of (a) p-mTOR, (b) p-4EBP1, (c) p-S6 and (d) p-ERK. Immunoreactivity of all signaling factors was detected in cytoplasmic of tumor cells. (a–d) Original magnification, ×100.

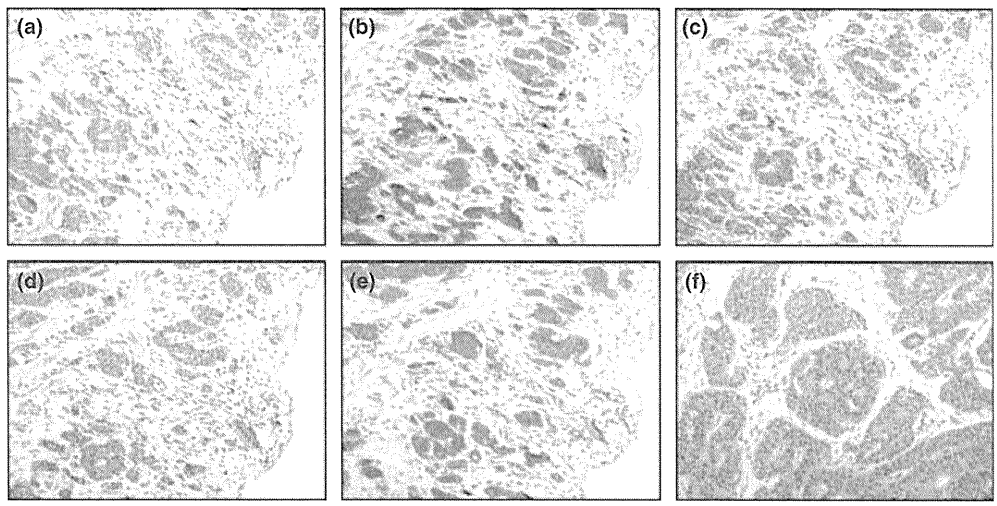

Fig. 3. Representative illustrations of immunohistochemistry of somatostatin receptor (sstr) subtypes and p-IGF-1R. Immunoreactivity of all sstr subtypes was detected in the membrane or cytoplasmic of the tumor cells. (a) sstr1; (b) sstr2A; (c) sstr2B; (d) sstr3; (e) sstr5; (f) p-IGF-1R. (a–f) Original magnification, ×100.

Multivariate analysis of clusters of immunoreactivity and clinicopathological characteristics of NET cases examined. We performed chi-squared tests in order to define the features of each clusters regarding the patterns of immunoreactivity of the factors examined. We first defined the features between Cluster I and Cluster II. Results of this analysis demonstrated that immunoreactivity of p-ERK, sstr2A and sstr5 did not show any significant differences among the three groups above, but that of p-mTOR (P = 0.038), p-4EBP1 (P = 0.026), p-S6 (P = 0.0066), sstr1 (P = 0.0066), sstr2B (P < 0.001), sstr5 (P = 0.0036),

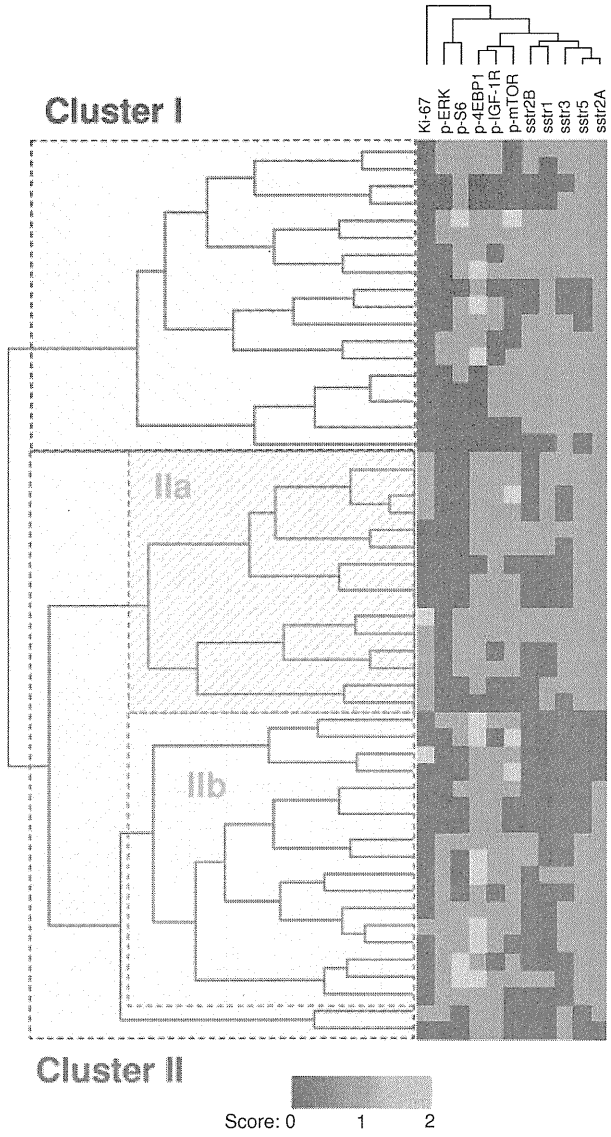

Fig. 4. Summary of hierarchical clustering analysis of the immunohistochemical data of 52 neuroendocrine tumor cases. The branch length represents the similarity between results obtained in this system. Neuroendocrine tumor cases in the present study were classified into the following three different groups according to the results: Cluster I, 18 cases; Cluster IIa, 15 cases; Cluster IIb, 17 cases. Two cases belonging to Cluster II were excluded because of the branch length. 4EBP1, eukaryotic initiation factor 4-binding protein 1; ERK, extracellular signal-regulated kinase; IGF-1R, insulin-like growth factor 1 receptor; mTOR, mammalian target of rapamycin; S6, ribosomal protein s6; sstr, somatostatin receptor.

p-IGF-1R ($P = 0.016$) and Ki-67 ($P = 0.018$) did show significant differences among the three clusters above (Table 5). These results indicated that the Cluster I cases ($n = 18$) were associated with expression of the sstr subtypes rather than the proteins in the intracellular signaling pathways. In contrast, the Cluster II cases ($n = 34$) were associated with relative abundance of p-mTOR, p-4EBP1 and p-S6, compared with the sstr subtypes above and higher proliferative activities. We then studied the correlation between clinicopathological features of individual cases and the clusters above using chi-squared tests, but there were no significant differences between the clusters of the patients examined (data not shown).

We subsequently performed chi-squared tests between Cluster IIa and Cluster IIb. Results showed that the Cluster IIa cases

Table 5. Summary of scoring of immunohistochemistry between Cluster I and II

	Cluster I ($n = 18$)	Cluster II ($n = 34$)	*P*-value
p-mTOR (Score 0 *vs* 1, 2)	8	25	**0.038**
p-4EBP1 (Score 0 *vs* 1, 2)	12	31	**0.026**
p-S6 (Score 0 *vs* 1, 2)	14	13	**0.0066**
p-ERK (Score 0 *vs* 1, 2)	7	11	0.64
sstr1, positive	14	13	**0.0066**
sstr2A, positive	18	30	0.13
sstr2B, positive	13	7	**<0.001**
sstr3, positive	15	14	**0.0036**
sstr5, positive	15	24	0.31
p-IGF-1R, positive	10	28	**0.016**
Ki-67 LI			
<2%	17	22	**0.018**
≥2%	1	12	

4EBP1, eukaryotic initiation factor 4-binding protein 1; ERK, extracellular signal-regulated kinase; IGF-1R, insulin-like growth factor 1 receptor; LI, labeling index; mTOR, mammalian target of rapamycin; S6, ribosomal protein s6; sstr, somatostatin receptor. The bold values indicate the statistical significance.

Table 6. Summary of scoring of immunohistochemistry between Cluster IIa and IIb

	Total ($n = 32$)	Cluster IIa ($n = 15$)	Cluster IIb ($n = 17$)	*P*-value
p-mTOR (Score 0 *vs* 1, 2)	25 (78.1%)	12	13	0.81
p-4EBP1 (Score 0 *vs* 1, 2)	30 (93.8%)	13	17	0.12
p-S6 (Score 0 *vs* 1, 2)	14 (43.8%)	4	10	0.067
p-ERK (Score 0 *vs* 1, 2)	11 (34.3%)	0	11	**<0.001**
sstr1, positive	13 (40.6%)	10	3	**0.0048**
sstr2A, positive	29 (90.6%)	15	14	0.087
sstr2B, positive	7 (21.9%)	4	3	0.54
sstr3, positive	12 (37.5%)	8	4	0.082
sstr5, positive	25 (78.1%)	15	10	**0.0049**
p-IGF-1R, positive	26 (81.3%)	12	14	0.84
Ki-67 LI				
<2%	20 (62.5%)	5	15	**0.0014**
≥2%	12 (37.5%)	10	2	

4EBP1, eukaryotic initiation factor 4-binding protein 1; ERK, extracellular signal-regulated kinase; IGF-1R, insulin-like growth factor 1 receptor; LI, labeling index; mTOR, mammalian target of rapamycin; S6, ribosomal protein s6; sstr, somatostatin receptor. The bold values indicate the statistical significance.

were associated with higher expression of sstr1 and 5 and higher proliferative status evaluated by Ki-67 immunohistochemistry (Table 6; $P = 0.0048$, 0.0049 and 0.0014, respectively). However, the Cluster IIb cases were associated with ERK activation ($P < 0.001$). Therefore, we then evaluated the correlation of the results with the clinicopathological features above and the results indicated that the status of age and their localization was significantly different between these clusters (Table 7; $P = 0.0078$ and 0.0043, respectively).

Effects of mTOR inhibitors on the cell proliferation in NET cell lines. Because the Cluster II cases were associated with the expression of p-mTOR and higher proliferative activities, we examined the effects of mTOR inhibitor, rapamycin, on cell proliferation using two NET cell lines, H727 and COLO. We performed a cell proliferation assay at a range of 10^{-9} to 10^{-7} M for 9 days (H727) or 3 days (COLO), and the results showed that there was a significant decrease in the cell number for 9 days in H727 and 3 days in COLO treated with rapamycin in a concentration-dependent manner (Fig. 5).

doi: 10.1111/j.1349-7006.2010.01657.x

Table 7. Characteristics of the clinicopathological findings of individual patients in Cluster IIa and IIb

	Total (n = 32)	Cluster IIa (n = 15)	Cluster IIb (n = 17)	P-value
Age (years)				
<60	23 (71.9%)	7	16	**0.0078**
≥60	9 (28.1%)	8	1	
Mean ± SD	52.7 ± 14.2	56.4 ± 17.4	49.4 ± 10.0	
Gender				
Male	22 (68.8%)	8	14	0.077
Female	10 (31.3%)	7	3	
Localization				
Foregut	9 (28.1%)	8	1	**0.0043**
Midgut	1 (3.1%)	1	0	
Hindgut	22 (68.8%)	6	16	
Lymph metastasis				
Presence	3 (9.4%)	3	0	0.053
Absence	29 (90.6%)	12	17	
Vascular invasion				
Presence	9 (28.1%)	6	3	0.16
Absence	23 (71.9%)	9	14	

The bold values indicate the statistical significance.

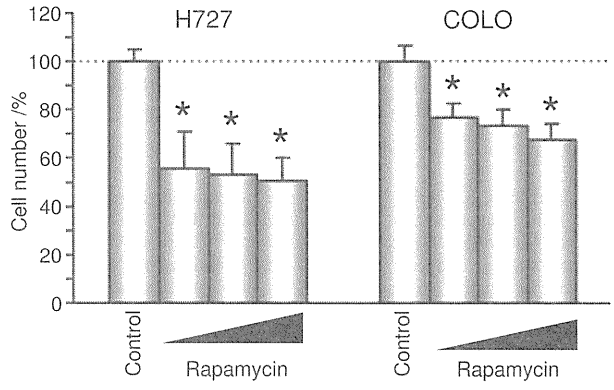

Fig. 5. Antitumor effects of rapamycin in neuroendocrine tumor cell lines in a concentration-dependent manner. Rapamycin, 10^{-9}, 10^{-8}, 10^{-7} M; H727, NCI-H727; COLO, COLO320-DM. All data are shown as mean (n = 6) ±SD. *P < 0.001 (*vs* Control).

Discussion

It is true that the main therapy of NET is surgical excision. Neuroendocrine tumor patients are generally considered resistant to traditional cytotoxic agents when they are in an advanced clinical stage.[21] In particular, the majority of NET cases arising in the foregut and hindgut, which were the predominant NET cases in Japan,[2] do not manifest clinically detectable endocrine manifestations and may be first detected at advanced clinical stages.[22] Somatostatin receptor subtypes have been demonstrated in the great majority of NET cases, including those arising in the foregut and hindgut, even at advanced clinical stages.[5] Octreotide is well known to inhibit the release of hormones and subsequently control symptoms in NET patients. Recently, a newly developed SOM230 (pasireotide; Novartis, St Louis, MO, USA), which could react with wider sstr subtypes, has been reported to be more effective in controlling cell proliferation and symptoms in preclinical studies.[23] In addition, some groups, including our laboratories, showed the antitumor effects of SSA in preclinical and clinical study.[5,24,25] However, its clinically effective antitumor activity has not necessarily been detected with octreotide alone, because the tumor response rate for octreotide represents <10%,[4,26] and thus, the antitumor activities of SSA have been controversial. Therefore, other modes of medical therapy have been in demand clinically, particularly for controlling tumor cell proliferation of non-functioning NET cases including those arising in the foregut and hindgut.

Other modes of intracellular signaling pathways have been reported to be involved in NET cases and among these pathways, in particular, mTOR activities have been shown to increase in NET cells, as a result of mutations of the tumor suppressor genes in the PI3K/Akt/mTOR pathway, rather than the genes encoding mTOR. For instance, the loss of heterozygosity of the *NF1* gene led to constitutive mTOR activation.[27] Neurofibromatosis type 1 (NF-1) is an autosomal dominant disorder clinically characterized by the presence of cutaneous and subcutaneous neurofibromas, café-au-lait spots and Lisch nodules. Neurofibromatosis type 1 appears to play a role as a tumor suppressor gene to function the *Ras* pathway.[28] Tumors associated with NF-1 include not only neurogenic neoplasms such as neurofibromas and neurofibrosarcomas, but also pheochromocytomas and NET, suggesting a broader role for *NF-1* as a tumor suppressor gene. However, the GI NET harboring *NF-1* genetic abnormalities often occurs in duodenal, ampullary NET and somatostatinomas. In addition, the presence of *NF-1* mutations in NET was reported in only 1–2% of cases.[16,29] However, it also awaits further investigations to clarify the possible involvement of *NF-1* genetic abnormalities in patients with NET. The overactivation of IGF-1R is also reported to be correlated with activation of the PI3K/Akt/mTOR pathway in NET cells.[13,30] von Wichert *et al.*[12] demonstrated that low-grade NET co-expressed IGF-1 and IGF-1R, and BON, a human pancreatic NET cell line, expressed functionally active IGF-1R and secreted IGF-1, which all suggest an autocrine action of this growth factor in NET. In addition, a Phase II clinical trial in which the IGF-1R monoclonal antibody is used for NET patients is in progress.[31] However, the immunohistochemical study of p-IGF-1R in human NET cases has not been previously reported. In addition, correlation of the sstr subtypes with the IGF-1R signaling pathway has also not been reported.

Therefore, in this study, we evaluated sstr subtypes, key factors in major signaling pathways under RTK and a potential therapeutic targeted RTK in NET cases using immunohistochemistry combined with hierarchical clustering analysis. Neuroendocrine tumors have been reported to be associated with specific patterns of sstr expression and sstr2 and sstr5 were predominant subtypes reported in Japanese NET patients.[5] Somatostatin receptor 1 and sstr3 are expressed less frequently and sstr4 is rarely expressed in NET as described above.[17,18] Results of our present immunohistochemical study were also consistent with those reported previously, and in particular, sstr2A and sstr5 were the most frequently detected sstr subtypes in these GI NET.[2,5] In addition, results of our present study also showed that the NET cases were basically classified into two different groups, Cluster I and II, and Cluster II was then further sub-classified into Cluster IIa and IIb. Between Cluster I and II, Cluster I was associated with a higher expression of the sstr subtypes, but there were no significant differences between these two clusters in the expression of sstr2A and sstr5. In addition, all Cluster IIa cases expressed sstr2A, but not Cluster IIb. Therefore, the status of the proliferative activity and lymph node metastasis was indeed associated with that of sstr2A and sstr5 expressions regardless of the status of p-IGF-1R immunoreactivity in the cases examined.

Shah *et al.*[14] also demonstrated a relative high abundance of p-endothelial growth factor receptor (p-EGFR) and p-ERK in NET cases using immunohistochemistry. In our present study, phosphorylated factors in the PI3K/Akt/mTOR pathway were also detected in many of the NET cases examined, but the cases associated with activated ERK were relatively low in number.

Possible reasons for the discrepancy between the report of Shah *et al.* and our present study might be due to differences of the sensitivities of the primary antibodies, or the majority of the localization (midgut *vs* hindgut) of the cases examined. In addition, results of our present study demonstrated that the cases belonging to Cluster IIb were associated with PI3K/Akt/mTOR and MEK/ERK pathways related to IGF-1R. These cases were associated with a relatively low proliferative status but may be treated with mTOR inhibitors/IGF-1R antagonists combined with MEK inhibitors, but further investigation is required for clarification.

Mammalian target of rapamycin inhibitors are macrolide antibiotics with potent immunosuppressive and antitumor activities. These agents bind immunophilin FK506-binding protein 12 (FKBP12), and this complex subsequently binds to mTOR, which inhibits downstream signaling pathways.[32,33] Recently, the antitumor activities of mTOR inhibitors have been extensively studied, and treatment of the mTOR inhibitors such as temsirolimus (CCI-779; Wyeth, Philadelphia, PA, USA) and everolimus (RAD001; Novartis, Basel, Switzerland) for advanced renal cell carcinoma after vascular endothelial growth factor receptor (VEGFR)-targeted therapy have been approved in Europe, the USA and Japan.[34,35] In NET patients, the effects of mTOR inhibitors have also been evaluated in both preclinical and clinical studies.[36,37] In the present study, we performed an *in vitro* study using NET cell lines in order to evaluate whether this classification has any relationship with sensitivity to the molecular target therapy. We examined the antitumor effects of rapamycin in NET cell lines, in which the PI3K/Akt/mTOR pathway was shown to be activated,[38,39] which suggests that the cases associated with overexpression of p-mTOR may be treated with mTOR inhibitors.

We subjected the results of the immunohistochemistry into hierarchical clustering analysis. This analysis is one of the multivariative statistical methods that identifies groups of samples that behave similarly or show similar characteristics.[20] Therefore, hierarchical clustering analysis following immunohistochemistry of different molecules may contribute to a potential new classification method according to biological features. Results of the present study revealed that NET cases were basically classified into the ''sstr subtypes expressing predominant'' group (Cluster I) and the ''activating signaling pathways predominant'' group (Cluster II), and the latter group was further sub-classified into the ''sstr expression with higher proliferative status predominant'' group, or Cluster IIa, and the ''activating ERK cascade predominant'' group, or Cluster IIb.

In conclusion, we are first to demonstrate the application of a novel classification method for non-functioning NET patients using hierarchical clustering analysis based on the immunohistochemical data of sstr subtypes, factors of a major signaling pathway under RTK and major RTK and clinicopathological factors of individual patients. It will be important to evaluate which group the cases with non-functioning NET belong to, and to determine the treatment of adequate drugs for individual NET patients.

Acknowledgments

The authors appreciate Dr Fumiaki Tezuka (Department of Pathology, Sendai Red Cross Hospital) for providing NET samples.

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mTOR expression and activity patterns in gastroenteropancreatic neuroendocrine tumours

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Abstract

Clinical trials indicate efficacy of drugs inhibiting the mammalian target of rapamycin (mTOR) in the treatment of gastroenteropancreatic neuroendocrine tumours (GEP-NET); however, information on detailed expression and activity patterns of mTOR in these tumours is sparse. We investigated the expression of mTOR and expression as well as phosphorylation of its downstream targets 4EBP1, S6K and eIF4E in a cohort of 99 human GEP-NET by immunohistochemistry. We correlated our findings with clinicopathological variables and patient prognosis. We found that 61, 93, 80, 69, 57 and 79% of GEP-NET were positive for mTOR, 4EBP1, cytoplasmic phospho-4EBP1 (p-4EBP1), nuclear p-4EBP1, phospho-S6K (p-S6K) and phospho-eIF4E (p-eIF4E) respectively. mTOR expression and activity were higher in foregut than in midgut tumours. In foregut tumours, expression of mTOR was higher when distant metastases were present ($P=0.035$). Strong mTOR activity was associated with higher proliferative capacity. In patients with stage IV midgut tumours, strong p-S6K expression was associated with poor disease-specific survival ($P=0.048$). In conclusion, mTOR shows considerable variations in expression and activity patterns in GEP-NET in dependence of tumour location and metastatic status. We hypothesise that these differences in mTOR expression and activity might possibly influence response to mTOR inhibitors.

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Introduction

With an incidence rate of about 2.5–5 cases per 100 000 person-years, gastroenteropancreatic neuroendocrine tumours (GEP-NET) are rather rare neoplasms when compared with adenocarcinomas of the same locations (Modlin *et al.* 2008). However, it is worth noting that the incidence of these tumours has risen tremendously over the last decades (Modlin *et al.* 2003, Yao *et al.* 2008*a*). Today, GEP-NET are treated in multidisciplinary approaches including surgery, biotherapy, chemotherapy as well as molecular targeted therapy (Plöckinger & Wiedenmann 2007, Oberg & Jelic 2008). Unfortunately, all improvements in the understanding and treatment of this disease have not resulted in significantly prolonged overall patient survival (Modlin *et al.* 2008), therefore novel treatment strategies for these tumours are still urgently needed.

Recently, the mammalian target of rapamycin (mTOR) inhibitors temsirolimus (Rini 2008) and everolimus (Sánchez-Fructuoso 2008) have entered late-phase clinical trials in a broad variety of solid human tumours. Both substances have been tested for their activity in phase II studies (Duran *et al.* 2006, Yao *et al.* 2008*b*, 2010) in a heterogeneous set of neuroendocrine neoplasms, and everolimus proved to be effective in the first place, especially in pancreatic

neuroendocrine carcinomas. Tissue-based predictive biomarkers for response to everolimus are currently lacking. However, expression of mTOR pathway components has been suggested as a predictive biomarker for response to temsirolimus (Duran *et al.* 2006).

The mTOR protein is a central component of two protein complexes intimately involved in carcinogenesis (Sabatini 2006). mTOR complex 1 (mTORC1), also containing raptor and mLST8, phosphorylates the eukaryotic translation initiation factor 4E-binding protein (4EBP1) and the ribosomal S6 kinase (S6K1). Phosphorylation of 4EBP1 in turn leads to a dissociation of the protein from eIF4E, an important regulator of translation, subsequently eIF4E gets phosphorylated and activated (Whalen *et al.* 1996). Activation of these factors, finally, leads to enhanced cancer cell growth, prolonged cancer cell survival and neoangiogenesis (Hay & Sonenberg 2004). mTORC1 itself is activated via the PI3K–AKT pathway partly through the deactivation of the tuberous sclerosis 1 (TSC1) and tuberous sclerosis 2 (TSC2) complexes (Gao *et al.* 2002). With respect to GEP-NET, this is interesting since patients with certain tumour syndromes with impaired TSC1/TSC2 function, such as tuberous sclerosis, are known to develop these neoplasms (Toumpanakis & Caplin 2008). The mTORC2, which does not contain raptor but rictor and mSin1, is less well understood (Sarbassov *et al.* 2005). However, there is evidence that this complex is able to activate AKT thereby inducing anti-apoptotic and pro-proliferative stimuli.

In this study, we aimed to investigate the expression and activity state of mTOR and its downstream targets 4EBP1, S6K and eIF4E in a large cohort of gastro-enteropancreatic neuroendocrine foregut and midgut tumours. We correlated our findings with clinicopathological variables and patient prognosis.

Patients, materials and methods

Patient characteristics

A total of 99 patients with GEP-NET of the foregut (47, 47.5%) and midgut (52, 52.5%), who received surgical treatment at the Charité University Hospital between 1983 and 2007, were included in the study. In detail, 9 (9.1%) tumours were gastric, 6 (6.1%) were duodenal, 31 (31.3%) were pancreatic, 3 (3%) were jejunal and 50 (50.5%) were ileal. In 70 cases, tissue from the primary lesion was available, 10 and 19 tissue specimens were from nodal and distant metastases respectively. In addition, in 33 cases, the primary tumours as well as nodal metastases were available for analysis. In 23 cases, the primary tumour and corresponding distant metastasis could be investigated. All cases were validated by immunohistochemistry in the routine diagnostic setting. By convention, antibodies against chromogranin A and synaptophysin were used to ensure neuroendocrine differentiation. If only one of the markers was positive, cluster of differentiation CD56 was stained in addition. Only cases with expression of two markers were designated as NETs. None of the patients included in this study had a hereditary syndrome, such as von Hippel–Lindau disease or multiple endocrine neoplasia nor were there familial cases without a known germline mutation. The mean age of patients with foregut tumours was 53.0 years at the time of the diagnosis. The mean age of patients with midgut tumours was 58.2 years. Of 99 patients, 51 (51.5%) were male. There was no association of sex distribution with tumour location in foregut or midgut. Follow-up data were available for almost all patients. However, since NET-related death occurred in the minority of patients with low-stage and low-grade tumours and since NETs of different locations are known to have significantly different survival (Plöckinger & Wiedenmann 2007), we decided to perform survival analysis exclusively in the homogenous group of midgut patients with stage IV disease. In this subgroup of 39 patients for whom data were available, 8 (20.5%) died of their disease after a mean follow-up time of 78.0 months. Those patients still alive at the endpoint of analysis were followed for a mean time of 48.3 months (range 3.5–210.7 months). We were able to gather treatment data in 21 of these 39 stage IV midgut patients. Of these, 11 received somatostatin receptor antagonists, 7 patients received no further treatment and 3 patients received other treatment regimens (including conventional chemotherapeutics).

NETs were re-graded and re-staged according to the novel consensus proposals for GEP-NET and according to the WHO (7th edn; Rindi *et al.* 2006, 2007, Sobin *et al.* 2009). The clinicopathological characteristics of the patients are given in Tables 1 and 2. The study has been approved by the Charité University Ethics Committee (EA1/06/2004).

Tissue

For the evaluation of mTOR, 4EBP1, phospho-4EBP1 (p-4EBP1), phospho-S6K (p-S6K) and phospho-eIF4E (p-eIF4E) expression, tissue microarrays were generated using a precision instrument (Beecher

Table 1 mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression in gastroenteropancreatic neuroendocrine tumours of the foregut and correlation with clinicopathological variables

Characteristics	All cases	mTOR negative	mTOR positive	*P* value	4EBP1 negative	4EBP1 positive	*P* value	p-4EBP1 cytoplasmic negative	p-4EBP1 cytoplasmic positive	*P* value	p-4EBP1 nuclear negative	p-4EBP1 nuclear positive	*P* value	p-S6K negative	p-S6K positive	*P* value	p-eIF4E negative	p-eIF4E positive	*P* value
All cases	47	14 (29.8%)	33 (70.2%)		1 (2.1%)	46 (97.9%)		2 (4.3%)	45 (95.7%)		11 (23.4%)	36 (76.6%)		10 (21.7%)	36 (78.3%)		7 (15.2%)	39 (84.8%)	
Stage				0.140[a]			0.884[a]			0.834[a]			0.359[a]			0.667[a]			0.216[a]
I	4 (8.9%)	2 (50%)	2 (50%)		0 (0%)	4 (100%)		0 (0%)	4 (100%)		1 (25%)	3 (75%)		2 (50%)	2 (50%)		2 (50%)	2 (50%)	
II	14 (31.1%)	4 (28.6%)	10 (71.4%)		0 (0%)	14 (100%)		0 (0%)	14 (100%)		2 (14.3%)	12 (85.7%)		0 (0%)	14 (100%)		0 (0%)	14 (100%)	
III	13 (28.9%)	7 (53.8%)	6 (46.2%)		1 (7.7%)	12 (92.3%)		2 (15.4%)	11 (84.6%)		3 (23.1%)	10 (76.9%)		3 (23.1%)	10 (76.9%)		1 (7.7%)	12 (92.3%)	
IV	14 (31.1%)	1 (7.1%)	13 (92.9%)		0 (0%)	14 (100%)		0 (0%)	14 (100%)		5 (35.7%)	9 (64.3%)		4 (28.6%)	10 (71.4%)		4 (28.6%)	10 (71.4%)	
Tumour stage				0.811[a]			0.717[a]			0.066[a]			0.848[a]			0.376[a]			0.700[a]
T1	7 (17.5%)	3 (42.9%)	4 (57.1%)		0 (0%)	7 (100%)		0 (0%)	7 (100%)		3 (42.9%)	4 (57.1%)		3 (42.8%)	4 (57.2%)		2 (33.3%)	4 (66.7%)	
T2	15 (37.5%)	4 (26.7%)	11 (73.3%)		0 (0%)	15 (100%)		0 (0%)	15 (100%)		2 (13.3%)	13 (86.7%)		2 (13.3%)	13 (87.7%)		(0%)	15 (100%)	
T3	5 (12.5%)	2 (40%)	3 (60%)		1 (20%)	4 (80%)		0 (0%)	5 (100%)		2 (40%)	3 (60%)		1 (20%)	4 (80%)		0 (0%)	5 (100%)	
T4	13 (32.5%)	4 (30.8%)	9 (69.2%)		0 (0%)	13 (100%)		2 (15.4%)	11 (84.6%)		4 (30.8%)	9 (69.2%)		2 (16.7%)	10 (83.3%)		3 (23.1%)	10 (76.9%)	
Nodal status				0.510[b]			0.386[b]			1.0[b]			0.724[b]			0.481[b]			1.0[b]
N0	28 (60.9%)	9 (32.1%)	19 (67.9%)		0 (0%)	28 (100%)		1 (3.6%)	27 (96.4%)		6 (21.4%)	22 (78.6%)		5 (18.5%)	22 (81.5%)		4 (14.3%)	24 (85.7%)	
N1	18 (39.1%)	5 (27.8%)	13 (72.2%)		1 (5.6%)	17 (94.4%)		1 (5.6%)	17 (94.4%)		5 (27.8%)	13 (72.2%)		5 (27.8%)	13 (72.2%)		3 (17.6%)	14 (82.4%)	
Metastasis				0.035[b]			1.0[b]			1.0[b]			0.225[b]			0.704[b]			0.190[b]
M0	32 (69.6%)	13 (40.6%)	19 (59.4%)		1 (3.1%)	31 (96.9%)		2 (6.3%)	30 (93.7%)		6 (20%)	24 (80%)		6 (19.3%)	25 (80.7%)		3 (9.7%)	28 (90.3%)	
M1	14 (30.4%)	1 (7.1%)	13 (92.9%)		0 (0%)	14 (100%)		0 (0%)	14 (100%)		5 (35.7%)	9 (64.3%)		4 (28.6%)	10 (71.4%)		4 (28.6%)	10 (71.4%)	
Grade				0.195[a]			/			0.622[a]			0.788[a]			0.263[a]			0.525[a]
G1	11 (35.5%)	3 (27.3%)	8 (72.7%)		0 (0%)	11 (100%)		0 (0%)	11 (100%)		2 (18.2%)	9 (81.8%)		3 (27.3%)	8 (72.7%)		2 (18.2%)	9 (81.8%)	
G2	16 (51.6%)	4 (25%)	12 (75%)		0 (0%)	16 (100%)		2 (12.5%)	14 (87.5%)		5 (31.2%)	11 (68.8%)		3 (18.8%)	13 (81.2%)		3 (18.8%)	13 (81.2%)	
G3	4 (12.9%)	3 (75%)	1 (25%)		0 (0%)	4 (100%)		0 (0%)	4 (100%)		0 (0%)	4 (100%)		0 (0%)	4 (100%)		0 (0%)	4 (100%)	

For one case, data on p-S6K and p-eIF4E expression were missing. Data on clinicopathological variables were missing for stage, T, N and M in a few cases in the respective subgroup analysis. For grade, only primary tumours were evaluated ($n=33$); in this subgroup, grading was not possible in two cases.

[a]χ^2 test for trends.

[b]Fisher's exact test.

Table 2 mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression in enteric midgut tumours and correlation with clinicopathological variables

Characteristics	All cases	mTOR negative	mTOR positive	*P* value	4EBP1 negative	4EBP1 positive	*P* value	p-4EBP1 cytoplasmic negative	p-4EBP1 cytoplasmic positive	*P* value	p-4EBP1 nuclear negative	p-4EBP1 nuclear positive	*P* value	p-S6K negative	p-S6K positive	*P* value	p-eIF4E negative	p-eIF4E positive	*P* value
All cases	52	24 (47.1%)	27 (52.9%)		6 (11.8%)	45 (88.2%)		18 (34.6%)	34 (65.4%)		20 (38.5%)	32 (61.5%)		32 (62.7%)	19 (37.3%)		13 (25.5%)	38 (74.5%)	
Stage				0.609[a]			0.538[a]			0.234[a]			0.347[a]			0.681[a]			0.801[a]
I	0 (0%)	/	/		/	/		/	/		/	/		/	/		/	/	
II	2 (3.8%)	0 (0%)	1 (100%)		0 (0%)	2 (100%)		0 (0%)	2 (100%)		0 (0%)	2 (100%)		1 (50%)	1 (50%)		0 (0%)	2 (100%)	
III	11 (21.2%)	7 (63.6%)	4 (36.4%)		1 (9.1%)	10 (90.9%)		3 (27.3%)	8 (72.7%)		4 (36.4%)	7 (63.6%)		7 (63.6%)	4 (36.4%)		3 (27.3%)	8 (72.7%)	
IV	39 (75%)	17 (43.6%)	22 (56.4%)		5 (13.2%)	33 (86.8%)		15 (38.5%)	24 (61.5%)		16 (41%)	23 (59%)		24 (63.2%)	14 (36.8%)		10 (26.3%)	28 (73.7%)	
Tumour stage				0.727[a]			0.617[a]			0.879[a]			0.366[a]			0.145[a]			0.897[a]
T1	1 (2%)	1 (100%)	0 (0%)		0 (0%)	1 (100%)		1 (100%)	0 (0%)		1 (100%)	0 (0%)		1 (100%)	0 (0%)		0 (0%)	1 (100%)	
T2	8 (16%)	3 (42.9%)	4 (57.1%)		0 (0%)	8 (100%)		2 (25%)	6 (75%)		1 (12.5%)	7 (87.5%)		5 (62.5%)	3 (37.5%)		1 (12.5%)	7 (87.5%)	
T3	21 (42%)	9 (47.4%)	12 (52.6%)		4 (19%)	17 (81%)		7 (33.3%)	14 (66.7%)		7 (33.3%)	14 (66.7%)		16 (76.2%)	5 (23.8%)		7 (33.3%)	14 (66.7%)	
T4	20 (40%)	9 (45%)	11 (55%)		2 (10.5%)	17 (89.5%)		7 (35%)	13 (65%)		9 (45%)	11 (55%)		10 (52.6%)	9 (47.4%)		3 (15.8%)	16 (84.2%)	
Nodal status				0.547[b]			0.572[b]			0.233[b]			0.127[b]			0.697[b]			1.0[b]
N0	8 (15.7%)	3 (42.9%)	4 (57.1%)		0 (0%)	8 (100%)		1 (12.5%)	7 (87.5%)		1 (12.5%)	7 (87.5%)		4 (50%)	4 (50%)		3 (37.5%)	5 (62.5%)	
N1	43 (84.3%)	21 (48.8%)	22 (51.2%)		6 (14.3%)	36 (85.7%)		17 (39.5%)	26 (60.5%)		19 (44.2%)	24 (55.8%)		27 (64.3%)	15 (35.7%)		10 (23.8%)	32 (76.2%)	
Metastasis				0.749[b]			1.0[b]			0.329[b]			0.524[b]			1.0[b]			1.0[b]
M0	14 (26.9%)	7 (53.8%)	6 (46.2%)		1 (7.1%)	13 (92.9%)		3 (21.4%)	11 (78.6%)		4 (28.6%)	10 (71.4%)		8 (57.1%)	6 (42.9%)		3 (21.4%)	11 (78.6%)	
M1	38 (73.1%)	17 (44.7%)	21 (55.3%)		5 (13.5%)	32 (86.5%)		15 (39.5%)	23 (60.5%)		16 (42.1%)	22 (57.9%)		24 (64.9%)	13 (35.1%)		10 (27%)	27 (73%)	
Grade				0.773[a]			0.366[a]			0.353[a]			0.669[a]			0.307[a]			0.280[b]
G1	13 (36.1%)	8 (61.5%)	5 (38.5%)		3 (23.1%)	10 (76.9%)		4 (30.8%)	9 (69.2%)		5 (38.5%)	8 (61.5%)		10 (76.9%)	3 (23.1%)		4 (30.8%)	9 (69.2%)	
G2	21 (58.3%)	8 (38.1%)	13 (61.9%)		3 (14.3%)	18 (85.7%)		10 (47.6%)	11 (52.4%)		9 (42.9%)	12 (57.1%)		13 (61.9%)	8 (38.1%)		4 (19%)	17 (81%)	
G3	2 (5.6%)	2 (100%)	0 (0%)		0 (0%)	2 (100%)		1 (50%)	1 (50%)		0 (0%)	2 (100%)		1 (50%)	1 (50%)		0 (0%)	2 (100%)	

For one case, data on mTOR, 4EBP1, p-S6K and p-eIF4E expression were missing. For some cases, data on clinicopathological variables were missing for stage, T, N and M in the respective subgroup analysis. For grade, only primary tumours were evaluated ($n=37$); in this subgroup, grading was not possible in one case.

[a]χ^2 test for trends.

[b]Fisher's exact test.

Instruments, Silver Spring, MD, USA). A representative tumour-bearing slide was selected for each case by a board certified pathologist with a special interest in GEP-NET pathology (WW). Typical tumour areas from the centre of the lesion as well as from the invasive margins were marked on the respective H&E slides. Subsequently, three tissue cylinders of 1.5 mm diameter were punched from each tumour-bearing donor block and transferred to a tissue microarray paraffin block. In addition, from every corresponding donor block, one conventional 2 μm paraffin section was cut for Ki-67 staining.

As normal reference control, ten cases of pancreatic tissue without significant pathology were investigated for the expression of the respective pathway components. Normal tissue was evaluated on conventional paraffin sections. Tissue was taken from patients with pancreatic NETs well away from the tumour.

Immunohistochemistry

Anti-mTOR antibody, anti-4EBP1, anti-4EBP1 phosphorylated at Thr70 (p-4EBP1), anti-eIF4E phosphorylated at Ser209 and anti-S6K phosphorylated at Thr389 antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). For immunohistochemistry, 3 μm paraffin sections were cut and incubated with anti-mTOR (1:50), anti-4EBP1 (1:50), anti-p-4EBP1 (1:25), anti-p-S6K (1:100) and anti-p-eIF4E (1:50) antibodies. The omission of the primary antibody served as negative control.

Ki-67 staining was performed in a Benchmark XT autostainer (Ventana, Tuscon, AZ, USA) according to the manufacturer's protocol.

Evaluation of staining of tissue slides

Staining of mTOR, 4EPB1, p-4EBP1, p-S6K and p-eIF4E in tumour tissue was scored by applying a semi-quantitative immunoreactivity scoring (IRS) system, as described previously (Darb-Esfahani *et al.* 2009). Briefly, category A documented the intensity of staining as 0 (no immunostaining), 1 (weak), 2 (moderate) and 3 (strong). Category B documented the percentage of immunoreactive cells as 0 (none), 1 (<10%), 2 (10–50%), 3 (51–80%) and 4 (>80%). Multiplication of categories A and B resulted in an IRS ranging from 0 to 12 for each individual case. The raw expression scores were used for correlation analysis. For correlation with clinicopathological variables, cases that showed any expression of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E (IRS 1–12) were scored as positive; cases without expression (IRS 0) were scored as negative.

Statistical analysis

Statistical analyses were carried out with SPSS 16.0 and GraphPad Prism 4.0. The significance of correlations between mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E staining patterns and clinicopathological data was tested by Fisher's exact test and χ^2 test for trends. The significance of correlations of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression scores in primary tumours and their corresponding lymph node and distant metastases was assessed by the Wilcoxon test for paired sample analysis. The correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression scores with each other and with proliferation indices was done by Spearman's

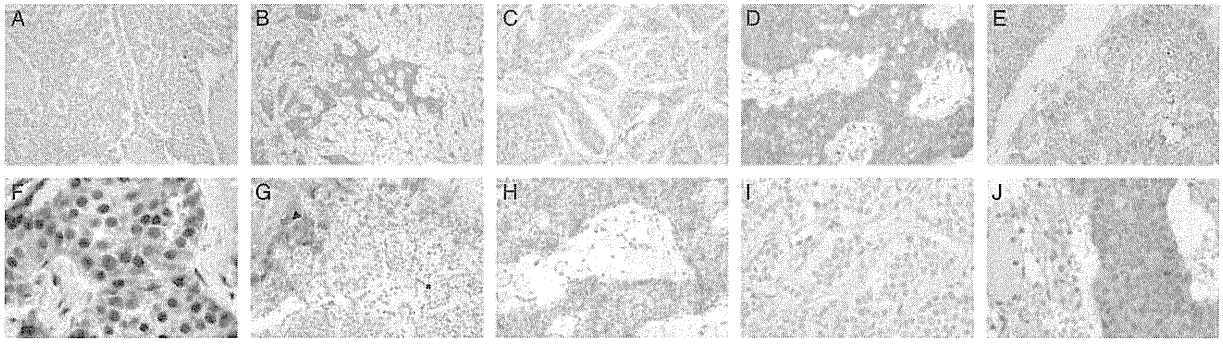

Figure 1 mTOR, 4EBP, p-4EBP1, p-S6K and p-eIF4E expression patterns in gastroenteropancreatic neuroendocrine tumours. (A/B) mTOR expression in GEP-NET. (A) An mTOR-negative tumour is shown. (B) Tumour with strong cytoplasmic mTOR positivity. (C/D) 4EBP1 in GEP-NET. Neuroendocrine tumours with weak (C) and strong (D) expression of 4EBP1. (E/F) p-4EBP1 expression in GEP-NET. (E) A tumour with strong cytoplasmic and without nuclear expression is depicted. In contrast, the tumour in (F) showed moderate cytoplasmic and strong nuclear positivity. (G/H) p-S6K in GEP-NET. While the tumour (arrow) in (G) was essentially negative for p-S6K, the tumour in (H) showed strong expression of the phosphorylated protein. Note strong expression in liver parenchyma (arrowhead in G). (I/J) p-eIF4E in GEP-NET. (I) A tumour without expression of p-eIF4E is depicted, while the tumour in (J) was scored as positive.

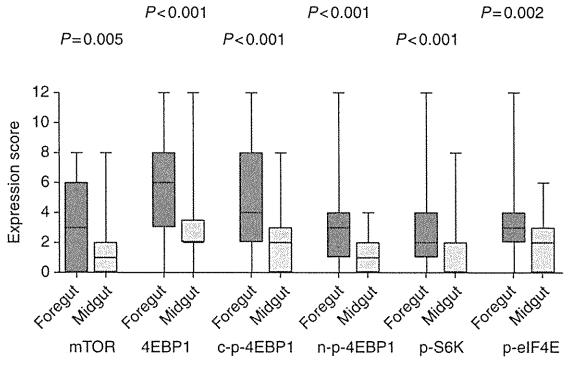

Figure 2 Expression of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E in dependence of tumour location. Expression of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E was higher in foregut than in midgut tumours. *P* values were calculated with the Mann–Whitney *U* test.

rank order correlation. Distribution of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression scores in dependence of tumour location was assessed by the Mann–Whitney *U* test. Differences in the percentages of Ki-67-positive cells in primary and metastatic tumours were investigated by the unpaired *t*-test and the Mann–Whitney *U* test.

The probability of differences in overall survival as a function of time was determined using the Kaplan–Meier method, with a log-rank test to probe for significance. P values <0.05 were considered significant.

Results

Expression patterns of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E in GEP-NET

Cytoplasmic mTOR expression was found in 60 (61.2%) out of 98 tumours available for analysis. No nuclear immunostaining was observed. The intensity of immunostaining ranged from weak to strong and was fairly homogenous throughout a given tumour (Fig. 1). mTOR expression was significantly higher in foregut tumours than in midgut tumours ($P=0.005$, Fig. 2); this was also true when stage was included in the analysis (data not shown). There was no significant difference between mTOR expression in gastric, duodenal and pancreatic tumours ($P=0.096$, data not shown). However, while gastric and pancreatic tumours showed the same prevalence of mTOR positivity (~67%), duodenal tumours were less likely to be positive (16.7%).

Cytoplasmic 4EBP1 immunopositivity was noted in 91 (92.9%) out of 98 tumours investigated (Fig. 1). A very faint nuclear staining was detected in some cases, which might correspond to the nuclear localisation of the phosphorylated protein (see below). However, nuclear staining was too weak to allow for a quantitative evaluation of this staining pattern. Expression of 4EBP1 was significantly higher in foregut tumours than in their midgut counterparts ($P<0.001$, Fig. 2), which again was independent from tumour stage (data not shown). No significant differences in expression were found when gastric, duodenal and pancreatic tumours were compared ($P=0.591$, data not shown).

Phosphorylated 4EBP1 was located either in the cytoplasm or in the nucleus in 79 (79.8%) and 68 (68.7%) cases respectively (Fig. 1). Both cytoplasmic and nuclear positivity were significantly more likely to be found in foregut than in midgut tumours (Fig. 2, $P<0.001$ for both correlations). This finding was also valid after differences in stage were taken into account (data not shown). Gastric, duodenal and pancreatic tumours showed no significant differences in the expression of cytoplasmic ($P=0.443$) and nuclear p-4EBP1 ($P=0.105$). However, pancreatic tumours showed a lower percentage of positive cases for nuclear expression (67.7%) when compared with duodenal (83.3%) and gastric (100%) tumours (data not shown).

Table 3 Correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression in gastroenteropancreatic neuroendocrine tumours

	mTOR score	4EBP1 score	Cytoplasmic p-4EBP1 score	Nuclear p-4EBP1 score	p-S6K score
4EBP1 score	$r=0.322$ $P=0.001$				
Cytoplasmic p-4EBP1 score	$r=0.402$ $P<0.001$	$r=0.632$ $P<0.001$			
Nuclear p-4EBP1 score	$r=0.260$ $P=0.009$	$r=0.406$ $P<0.001$	$r=0.642$ $P<0.001$		
p-S6K score	$r=0.187$ $P=0.067$	$r=0.443$ $P<0.001$	$r=0.281$ $P=0.005$	$r=0.239$ $P=0.019$	
p-eIF4E score	$r=0.346$ $P=0.001$	$r=0.646$ $P<0.001$	$r=0.259$ $P=0.010$	$r=0.162$ $P=0.113$	$r=0.374$ $P<0.001$

Phosphorylated S6K (p-S6K) was exclusively found in the cytoplasm of tumour cells (Fig. 1). In total, 56.7% of tumours were positive for activated S6K to varying degrees (Tables 1 and 2). Similar to mTOR and 4EBP4, p-S6K expression was higher in foregut than in midgut tumours ($P<0.001$, Fig. 2) in a stage-independent manner. There was no significant difference in the expression of p-S6K between gastric, duodenal and pancreatic tumours ($P=0.786$, data not shown).

p-eIF4E was observed in 79.4% of tumours and varied considerably from case to case (Fig. 1, Tables 1 and 2). Again, expression was significantly higher in tumours from foregut when compared with tumours from midgut origin ($P=0.002$, Fig. 2). With respect to specific foregut locations, the number of positive cases did not show a relevant variation ($P=0.983$, data not shown).

Overall mTOR expression significantly correlated with 4EBP1, cytoplasmic and nuclear p-4EBP1 expression as well as with p-eIF4E expression ($P<0.01$ for all comparisons). The correlation coefficients (r) indicated a modest to fairly strong degree of interaction (Table 3). mTOR was associated with p-S6K as well; however, the association was weak ($r=0.187$) and failed to show statistical significance ($P=0.067$, Table 3).

As normal reference control, mTOR pathway component expression was investigated in a set of normal pancreatic tissues including adjacent stromal and inflammatory cells. These stainings revealed stable expression of several of the proteins in a distinct set of normal cells (e.g. lymphocytes). The respective results are summarised in Supplementary Table 1, see section on supplementary data given at the end of this article.

Correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression with proliferation indices

Foregut tumours showed a higher proliferative activity than midgut tumours (mean foregut: 11% Ki-67-positive cells, mean midgut: 5% Ki-67-positive cells, $P=0.002$). This was also found when only stage IV tumours were compared ($P<0.001$).

By trend, Ki-67 staining was higher in nodal (mean primary: 3.1%, mean nodal metastasis: 4.2%) and distant metastases (mean primary: 3.3%, mean distant metastasis: 8.3%) when compared with the corresponding primary tumours. These differences were statistically significant in parametric tests for both comparisons ($P<0.001$) but only for the comparison of primary tumour and distant metastasis in non-parametric tests ($P=0.024$).

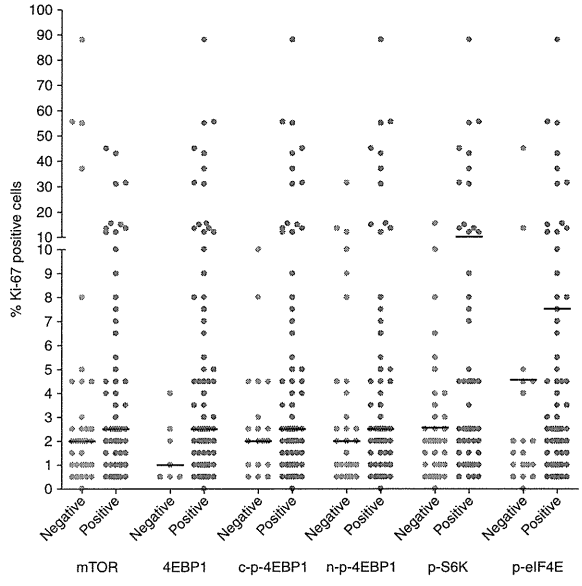

Figure 3 Proliferative activity in dependence of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression in GEP-NET. Proliferative activity was higher in those tumours with stronger expression of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E (details: see text).

Overall mTOR positivity was slightly but significantly higher in tumours with higher proliferative capacity ($r=0.213$, $P=0.038$). This correlation was also found for the expression of phosphorylated cytoplasmic and nuclear 4EBP1 ($r=0.238$, $P=0.020$ and $r=0.262$, $P=0.010$ respectively). Expression of 4EBP1 showed an even higher degree of correlation ($r=0.463$, $P<0.001$; Fig. 3). In addition, p-S6K ($r=0.364$, $P<0.001$) as well as p-eIF4E ($r=0.273$, $P=0.008$) expression was associated with higher proliferative capacity, as well (Fig. 3).

Correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression with clinicopathological variables

In foregut, mTOR expression was significantly higher in tumours with distant metastasis ($P=0.035$; Table 1). No other correlations of the expression of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E with clinicopathological variables in either foregut or midgut tumours were evident (Tables 1 and 2).

Correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression in the primary tumour and in corresponding lymph node and distant metastases

We investigated the expression of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E in matched pairs of

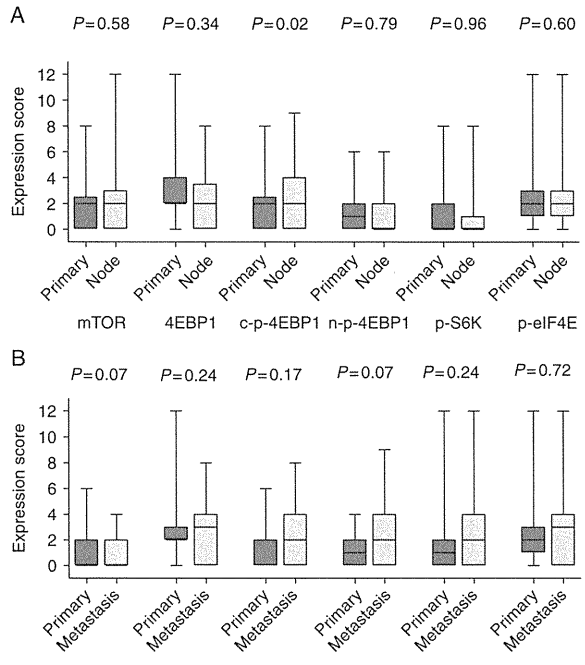

Figure 4 Correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression in primary tumours and corresponding lymph node and distant metastases. While mTOR expression is a bit lower in distant metastatic tumours when compared with the corresponding primaries, activation levels of 4EBP1 are usually higher in nodal and distant metastases and expression levels of p-S6K and p-eIF4E were higher in distant metastases. *P* values were calculated with the Wilcoxon test.

primary tumours, nodal and distant metastases of GEP-NET (Fig. 4). There was a tendency towards lower mTOR expression in distant metastasis when compared with the respective primary tumours; however, this correlation was only of borderline significance ($P=0.07$). In addition, metastatic nodal (only cytoplasmic p-4EBP1) and distant tumour seeds usually showed slightly higher expression of phosphorylated 4EBP1, S6K and eIF4E when compared with the corresponding primary tumour, indicating higher activity of the mTOR pathway in metastatic tumours. However, this association was only found to be significant for cytoplasmic p-4EBP1 and nodal spread ($P=0.02$) and was of borderline significance for nuclear p-4EBP1 and distant spread ($P=0.07$).

Correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression with survival

A probatory survival analysis in the homogenous subgroup of patients with stage IV midgut tumours ($n=39$) revealed that neither mTOR ($P=0.329$) nor 4EBP1 ($P=0.186$) or p-eIF4E ($P=0.521$) expression had an impact on NET-related death in univariate survival analysis in this group of patients (Fig. 5). Those patients whose tumours showed cytoplasmic p-4EBP1 expression had a trend towards longer disease-specific survival than those patients without activation of 4EBP1 ($P=0.055$). Interestingly, patients with activated S6K in their tumours had a significantly shortened disease-specific survival ($P=0.048$, Fig. 5). Neither grade ($P=0.764$) as a correlate for tumour aggressiveness nor treatment ($P=0.148$) had an impact on survival in this stage IV midgut patient cohort.

Discussion

In this study, we report a differential expression of mTOR, 4EBP1, phosphorylated 4EBP1, phosphorylated S6K and phosphorylated eIF4E in a large cohort of GEP-NET. Expression levels of mTOR as well as activation of its downstream targets were higher in foregut tumours than in midgut tumours, indicating a higher activity of the mTOR pathway in the former. This increase in activity was accompanied by a higher proliferative capacity of foregut tumours when compared with midgut tumours. Foregut tumours with distant metastases showed strong mTOR expression, and metastatic tumours in general showed slightly higher mTOR pathway activation indicated by enhanced phosphorylation of 4EBP1 as well as by enhanced phosphorylation of S6K and eIF4E. Interestingly, those stage IV midgut patients with activated S6K had a reduced disease-specific survival, while this was not true for other downstream effectors or mTOR itself.

The detection of p-4EBP1 in the nucleus by us and other groups both *in vitro* and *in vivo* is interesting (Zhou *et al.* 2004, Castellvi *et al.* 2006, Rojo *et al.* 2007, Rong *et al.* 2008). It has been demonstrated that the target of 4EBP1, eIF4E, has functions as a nuclear regulator of the export of several RNAs involved in proliferation and cell growth (Culjkovic *et al.* 2007). The presence of 4EBP1 in the nucleus has been proposed to provide a means to regulate the release of eIF4E from the nucleus and may thus prevent the untimely export of eIF4E bound mRNAs (Missiaglia *et al.* 2010). The relevance of this mechanism with respect to carcinogenesis has to be elucidated.

Recently, researchers have begun to focus on the mTOR pathway in GEP-NET, since treatment of metastasized NETs with the mTOR inhibitor everolimus in combination with octreotide showed promising results in phase II clinical studies (Yao *et al.* 2008*b*, 2010). In addition, the mTOR pathway plays a central role in the tumourigenesis of familial cases as well as in the sporadic cases of NETs. The notion that this pathway is of importance in this tumour entity has

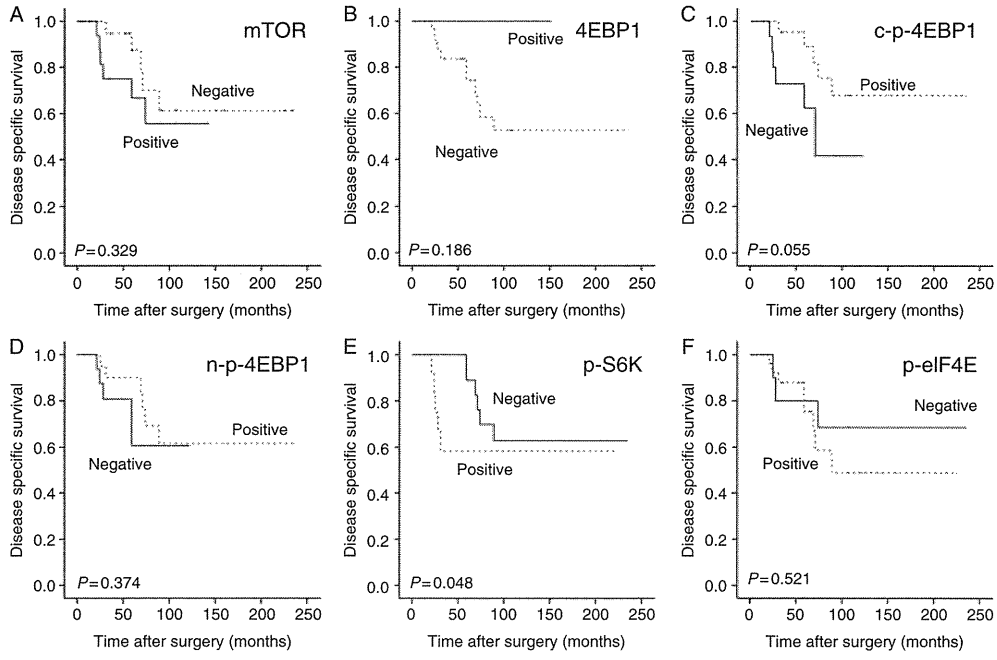

Figure 5 Correlation of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E expression with survival in stage IV midgut NET. No significant differences in disease-specific survival were observed in dependence of mTOR (A) and 4EBP1 (B), cytoplasmic (C) and nuclear (D) p-4EBP1 as well as p-eIF4E (F) positivity. In contrast, patients whose tumours expressed phosphorylated S6K (E) had a reduced disease-specific survival time. *P* values were calculated with a log-rank test.

further been substantiated by results of a high-throughput RNA expression analysis of pancreatic NETs in which the upstream inhibitors of mTOR, TSC2 and PTEN were found to be downregulated (Missiaglia *et al.* 2010). In addition, mTOR inhibition by rapamycin has been shown to significantly reduce NETs cell growth *in vitro* and *in vivo* (Moreno *et al.* 2008). This might be due to an induction of growth arrest in G_0/G_1 phase and enhanced apoptosis (Zitzmann *et al.* 2007). Furthermore, it has been proposed that deactivation of the AKT–mTOR kinase axis is responsible for this effect (Grozinsky-Glasberg *et al.* 2008). These *in vitro* results are in line with our findings that mTOR expression as well as downstream activation of 4EBP1, eIF4E and S6K correlates with proliferation in GEP-NET.

Most recently, in analogy to our work in GEP-NET, a large study on the expression of mTOR pathway components in lung NETs has been published in this journal (Righi *et al.* 2010). The authors reported an overexpression of p-4EBP1 in high-grade tumours, in contrast to p-mTOR and p-S6K, which were strongly expressed in low-grade tumours. In addition, in one recently published study on gastrointestinal NETs, phosphorylated mTOR, p-4EBP1 and p-S6K expression as well as several other factors were used to subclassify NET into novel potentially biological important subgroups (Iida *et al.* 2010). However, a correlation of the respective proteins with clinico-pathological variables and outcome has not been reported. Besides this, just one study on the expression of p-mTOR, which included only 20 GEP-NET (Shida *et al.* 2010) and in which the authors reported enhanced p-mTOR expression in poorly differentiated tumours, has been published. In our study, we did not find a straightforward correlation of either grouped mTOR expression or mTOR activity (as indicated by phosphorylation of 4EBP1) with tumour grade. However, we found an association of the expression of these proteins with the proliferation index, which in the novel grading scheme for GEP-NET is the central classifier for tumour grade.

mTOR expression and activity have been evaluated in a broad variety of human tumours, including most of the major tumour types, namely endometrial (Darb-Esfahani *et al.* 2009), esophageal (Boone *et al.* 2008), renal (Campbell *et al.* 2008), colorectal (Tampellini *et al.* 2007), prostate (Kremer *et al.* 2006), liver (Sahin *et al.* 2004), breast (Zhou *et al.* 2004, Rojo *et al.* 2007), lung (Anagnostou *et al.* 2009) and ovarian (Noske *et al.* 2008) cancer as well as glioblastoma (Pelloski *et al.* 2006). In all tumour entities, mTOR was either upregulated and/or activated in the tumour tissue when compared with the

corresponding tissue of origin. In addition, in some tumour entities, mTOR activity was linked to compromised patient prognosis. However, an association of the activated mTOR pathway with a better patient prognosis has been reported (Noske *et al.* 2008, Anagnostou *et al.* 2009) as well. In one study on bronchial NETs, no prognostic impact of mTOR pathway components was reported (Righi *et al.* 2010). We found that although mTOR expression itself was not associated with differences in patient prognosis, the detection of activated S6K confers a poor prognosis in stage IV midgut NETs. However, since this very homogenous subgroup of patients comprised only 39 cases, our results with respect to a possible impact of p-S6K positivity on survival must clearly be confirmed in much larger study cohorts.

In summary, we found that expression and activity of mTOR were strongly dependent on primary tumour location and metastatic status in GEP-NET. Expression as well as activation of mTOR pathway components was associated with enhanced proliferative capacity. Since everolimus, a small molecule targeting mTOR, proved to be effective in this tumour type and since it has been shown that response to other mTOR inhibitors may vary in dependence of expression and/or activity of the target, we suggest an investigation of mTOR expression profiles and phosphorylation of downstream targets in future clinical trials with this inhibitor.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1677/ERC-10-0126.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported by a research stipend (Ernst von Leyden-Stipendium 2009) to A Kasajima by the Berliner Krebsgesellschaft.

Acknowledgements

We like to thank Lisa Glanz, Petra Wachs, Sylwia Handzik and Katsuhiko Ono for their excellent technical assistance.

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Received in final form 11 November 2010
Accepted 13 December 2010
Made available online as an Accepted Preprint
13 December 2010

がん診療ガイドラインの作成（新規・更新）と公開の維持および その在り方に関する研究
平成21年度～平成23年度

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研究要旨
大腸癌治療ガイドライン2005年版（初版）の発刊後、平成21年7月に改訂版（2009年版）を発刊した。改訂版では重要事項に関するCQを導入し、推奨度も提示した。その後、化学療法の薬剤の保険適応の変更があったため、平成22年7月に2010年版を発刊し、ホームページ上に公開した。また、2010年版の英語版を作成し、IJCO誌に掲載予定である。現在、大腸癌治療ガイドラインの利用状況を評価するwebアンケートを実施中である。

A．研究目的
大腸癌治療ガイドラインは2005年に初版が発刊されたが、その改訂版を発刊することを目的とした。さらに、本邦の大腸癌治療を海外に向けて発信するために、大腸癌治療ガイドラインの英語版を発刊することを目的とした。また、ガイドラインの利用状況を把握するため、インターネットによる全国的なアンケート調査を行うことを目的とした。

B．研究方法
1．大腸癌研究会のガイドライン委員会で改訂版を作成した。
2．大腸癌治療ガイドラインの英語版を作成した。
3．ガイドラインの利用状況についてwebアンケート調査を開始した。対象は大腸癌研究会の施設会員に限らず、全国的な規模の調査とした。
（倫理面への配慮）
該当無し。

C．研究結果
1．平成17年に大腸癌治療ガイドライン2005年版（初版）が発刊された後、平成21年7月に大腸癌治療ガイドライン2009年版（第2版）を発刊した。初版では、クリニカルクエスチョン（CQ）が掲載されていなかったが、改訂版ではCQを導入し、重要と考えられる項目についてCQを付記した。さらに、CQに対する推奨文に推奨度も提示した。
2．大腸癌治療ガイドライン2009年版の発刊後、化学療法における薬剤の保険適応などが変更されたため、平成22年7月に大腸癌治療ガイドライン2010年版（第3版）を発刊した。
3．ガイドライン2010年版の英語版をInternational Journal of Clinical Oncology誌に投稿し、平成23年8月にacceptされた。平成24年に雑誌に掲載される予定である。
4．大腸癌治療ガイドラインの利用状況に関するwebアンケートを現在施行中である。
5．次回改訂に向けたクリニカルクエスチョン（CQ）（案）に対する意見をH23年12月まで収集中である。

D．考察
大腸癌の化学療法では、様々な新規薬剤が導入されたが、欧米では使用できても、本邦では使用できないものも存在する。従って、ガイドライン作成時には、本邦における薬剤の使用可能状況を十分考慮する必要があり、これらの状況に変化があった場合には、迅速に対応する必要がある。実際、今回、大腸癌治療ガイドライン2009年度版が発刊された後、本邦の保険適応が変更された。このため、最新の情報を提供するため、大腸癌治療ガイドライン2009年度版が発刊されて1年しか経過していなかったが、平成22年7月には、大腸癌治療ガイドライン2010年度版を発刊した。さらに、本邦の大腸癌治療指針を海外にも発信できるように大腸癌治療ガイドラインの英語版を作成し、International Journal of Clinical Oncology誌に掲載予定となっている。また、現在ガイドラインの使用状況あるいは記載内容などに関する意見などについてweb上でアンケート調査を実施中であり、これによりガイドラインの評価を行うことで、今後のガイドライン改訂版の作成に有効的に生かしていくことができると考えられる。

E．結論
大腸癌診療ガイドライン2009年版および2010年度版を作成した。また、ガイドラインの英語版を作成し、平成24年に英文雑誌（International Journal of Clinical Oncology）に掲載される予定である。ガイドライン評価のためのアンケート調査を実施中であり、今後の改訂作業に結果を反映させる予定である。

F．健康危険情報
特記事項無し。

G．研究発表
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H．知的財産権の出願・登録状況
1．特許取得
該当無し。
2．実用新案登録
該当無し。
3．その他
特記事項無し。