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ORIGINAL ARTICLE

Expression of an activated mammalian target of rapamycin (mTOR) in gastroenteropancreatic neuroendocrine tumors

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Abstract
Aims Gastroenteropancreatic neuroendocrine tumors are rare, and the current WHO classification divides this tumor entity into well-differentiated (neuro)endocrine tumors, well-differentiated (neuro)endocrine carcinomas, and poorly differentiated (neuro)endocrine carcinomas. Poorly differentiated (neuro)endocrine carcinoma is extremely aggressive, and no appropriate therapeutic approach has been established. The mammalian target of rapamycin (mTOR), an important regulator of cell proliferation and protein translation, is activated in various malignancies. Recent phase II trial has revealed the efficacy of mTOR inhibitor (RAD001; everolimus) against low-to-intermediate grade neuroendocrine tumors. However, the beneficial role of mTOR inhibitor against poorly neuroendocrine carcinoma remains uncertain. The purpose of the present study was to determine the activation of mTOR in gastropancreatic neuroendocrine tumors, especially in poorly differentiated neuroendocrine carcinomas.
Methods Expression of p-mTOR(Ser2448) was assessed by immunohistochemistry in 20 gastropancreatic neuroendocrine tumors (seven well-differentiated neuroendocrine tumors, four well-differentiated neuroendocrine carcinomas, and nine poorly differentiated neuroendocrine carcinomas). Double immunohistochemistry was performed with p-Akt for patients with high p-mTOR expression.
Results Expression of mTOR was seen in 9 (45%) of 20 gastroenteropancreatic neuroendocrine tumors. High expression of p-mTOR was seen in 6 (67%) of 9 poorly differentiated neuroendocrine carcinomas which was higher than the expression rate of well-differentiated neuroendocrine tumors and carcinomas, 3 (27%) of 11. All large cell neuroendocrine carcinomas showed high p-mTOR expression. Some tumor cells showed positive staining for p-mTOR co-expressed p-Akt.
Conclusions High expression rate of p-mTOR in poorly differentiated neuroendocrine carcinomas (large-cell type) may suggest the potential role of mTOR inhibitors as effective therapeutic agents for this highly malignant disease.

Keywords mTOR · Gastroenteropancreatic · Neuroendocrine · Poorly differentiated

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Introduction

Neuroendocrine tumors originate from neuroendocrine cells distributed throughout the body. Gastroenteropancreatic neuroendocrine tumors are fairly rare, and the

treatment for this disease is still not established. Gastroenteropancreatic neuroendocrine tumors are classified according to the WHO classification into well-differentiated (neuro)endocrine tumors and carcinomas, or poorly differentiated (neuro)endocrine carcinomas [1]. Poorly differentiated neuroendocrine carcinomas are rare but highly malignant with an extremely poor prognosis [2, 3]. To date, there have been no appropriate and effective therapies against this highly malignant tumor.

The mammalian target of rapamycin (mTOR) is a conserved serine/threonine kinase that plays an important role in cellular growth and homeostasis. Its regulation is frequently altered in various tumors. mTOR is activated by phosphorylation through Akt via the phosphatidylinositol 3-kinase/AKT signaling pathway at Ser^{2448} and by autophosphorylation at Ser^{2481} site [4, 5]. Owing to its key function in cellular growth, mTOR is currently under investigation as a potential target for anticancer therapy and its effect has been proved in renal cell carcinoma in a recent phase III trial [6]. There are several mTOR inhibitors available today, i.e., rapamycin (sirolimus) and its analogs CCI-779 (temsirolimus), AP23573, and RAD001 (everolimus). Recently, mTOR inhibitor, RAD001 (Everolimus), showed promising antitumor activity against low-to-intermediate grade neuroendocrine tumors [7]. However, the effect of mTOR inhibitors against poorly differentiated neuroendocrine tumors has not been determined to date. Furthermore, the expression of activated mTOR in gastropancreatic neuroendocrine tumors has not been determined by clinical samples either.

The purpose of the present study was to evaluate the expression of activated mTOR on gastropancreatic neuroendocrine tumors, especially in poorly differentiated neuroendocrine carcinomas.

Materials and methods

Patients and tissues

Formalin-fixed and paraffin-embedded samples of gastroenteropancreatic neuroendocrine tumors ($n = 20$) were obtained from Department of General Surgery, Chiba University Hospital, Japan, from 1999 to 2007. Patients who underwent neoadjuvant therapy and with carcinomas besides gastroenteropancreatic carcinomas were excluded. Written informed consent was obtained from each patient. Pathologic diagnosis was done based on the WHO classification [1]. All tumors were categorized into three groups: well-differentiated neuroendocrine tumor (carcinoid, $n = 7$), well-differentiated neuroendocrine carcinoma ($n = 4$), and poorly differentiated neuroendocrine carcinoma ($n = 9$). Furthermore, poorly differentiated neuroendocrine carcinomas were subdivided into small-cell type ($n = 3$) and large-cell type ($n = 6$) on the basis of histological findings using the criteria commonly applied to pulmonary neuroendocrine carcinomas [8].

Immunohistochemistry

All tumors were examined by immunohistochemistry. Sections (4 μm) were cut from formalin-fixed paraffin-embedded tissues and placed on salinized slides. The antibodies used were as follows: rabbit monoclonal p-mTOR antibody (Phospho-mTOR, Ser^{2448}, 49F9, dilution 1:50; Cell Signaling Technology) and rabbit monoclonal p-Akt antibody (Phospho-Akt, Ser^{473}, 736E11, dilution 1:50; Cell Signaling Technology). Staining was done by the labeled streptoavidin–biotin–peroxidase method using LSAB + system-HRP kit (DakoCytomation) and microwave antigen retrieval for 10 min. The staining pattern was scored as follows: −, no staining or <5% of the tumor cells positive; +, occasional weak staining; ++, moderate staining; and +++, intense staining. For scores ++ and +++, more than 10% of the tumor cells needed to be positive.

Double immunohistochemical staining was done for p-mTOR and p-Akt in some selected cases. For color development, diaminobenzidine (DAB) was used for p-Akt, and AEC (Dakocytomation; Code K3436) was used for p-mTOR.

Statistical analysis

All analyses were performed using SPSS 14.0 software (SPSS Inc., Chicago, IL, USA). Fisher's test was applied for comparisons. Results were considered significant when $P < 0.05$ was obtained.

Results

A total of 20 gastropancreatic neuroendocrine tumor patients were included in the study. Clinicopathological features of the 20 patients are listed in Table 1.

Expression of p-mTOR in gastroenteropancreatic neuroendocrine tumors

For the evaluation of p-mTOR, − and + were considered low-expression group, ++ and +++ were considered high expression group. In the present study, 9 (45%) of 20 gastroenteropancreatic neuroendocrine tumors showed high expression for p-mTOR. p-mTOR was considered positive when cytoplasmic and/or membranous staining were observed (Fig. 1). The details of the immunohistochemical staining results are listed in Table 2. Three (27%) of 11

Table 1 Clinicopathological features of the gastroenteropancreatic NET patients

Case	Age/sex	Histology	Organ	Stage	Treatment	Prognosis (months)
1	58/F	NEC (large)	Rectum	III	Surgery + CT	NED (50)
2	69/M	NEC (small)	Rectum	III	Surgery + CT	DOD (3)
3	78/M	NEC (small)	Colon	IV	None	DOD (1)
4	79/F	NEC (large)	Rectum	III	Surgery + CT	NED (65)
5	47/F	NEC (large)	Colon	IV	Surgery + CT	DOD (6)
6	84/F	NEC (large)	Rectum	IV	Surgery + CT	AWD (36)
7	66/M	NEC (large)	Pancreas	III	Surgery + CT	AWD (13)
8	19/F	NEC (small)	Pancreas	IV	Surgery	DOD (2)
9	60/M	NEC (large)	Pancreas	IV	CT	DOD (7)
10	61/M	NEC (well)	Pancreas	IV	Surgery + CT	AWD (19)
11	69/M	NEC (well)	Pancreas	I	Surgery	NED (33)
12	67/F	NEC (well)	Pancreas	I	Surgery	NED (25)
13	59/F	NEC (well)	Pancreas	I	Surgery	NED (73)
14	55/F	NEC (well)	Pancreas	I	Surgery	NED (67)
15	33/F	NEC (well)	Duodenum	IV	Surgery + TACE	AWD (59)
16	57/M	NEC (well)	Pancreas	I	Surgery	NED (7)
17	44/F	NET (well)	Pancreas	I	Surgery	NED (55)
18	62/F	Carcinoid	Rectum	I	EMR	NED (41)
19	76/M	Carcinoid	Rectum	I	EMR	NED (52)
20	57/F	Carcinoid	Duodenum	I	Surgery	NED (34)

F female, *M* male, *CT* chemotherapy, *RT* radiation, *EMR* endoscopic mucosal resection, *TACE* transarterial chemoembolization, *NED* no evidence of disease, *AWD* alive with disease, *DOD* dead of disease, *NEC* neuroendocrine carcinoma, *NET* neuroendocrine tumor

well-differentiated neuroendocrine tumor and carcinoma patients showed high expression to p-mTOR. With regard to poorly differentiated neuroendocrine carcinoma, six (67%) of nine patients showed high expression to p-mTOR (Table 3). Though it did not reach statistical significance, poorly differentiated neuroendocrine carcinoma patients showed a tendency of higher expression of p-mTOR than well-differentiated neuroendocrine tumor and carcinoma patients ($P = 0.095$). All large cell carcinoma patients showed high expression to p-mTOR, however, none of the small cell carcinoma patients showed high expression to p-mTOR (Table 4).

Co-localization of p-mTOR and p-Akt

Double immunohistochemical staining reveled that in some tumor cells which showed positive staining for p-mTOR also showed positive for p-Akt. The staining of p-Akt was seen in the cytoplasm and nucleus (Fig. 1, arrow indicated).

Discussion

Gastroenteropancreatic neuroendocrine tumors are rare diseases that present many clinical symptoms. They secrete various kinds of neuroamines and peptides that cause obvious clinical syndromes, including carcinoid syndrome. Recently, a phase II study revealed the efficacy of RAD001 (Everolimus; mTOR inhibitor) against low-to-intermediate grade neuroendocrine tumors [7]. The WHO classification divides gastroenteropancreatic neuroendocrine tumors into three categories; well-differentiated (neuro)endocrine tumors, well-differentiated (neuro)endocrine carcinomas, and poorly differentiated (neuro)endocrine carcinomas [1]. Low-to-intermediate grade neuroendocrine tumors are equal to well-differentiated neuroendocrine tumors and carcinomas. Among these tumors, although the frequency is rare, poorly differentiated neuroendocrine carcinomas are extremely aggressive, and neither chemotherapeutic nor radiotherapeutic approaches are effective [9]. Appropriate therapeutic strategy against this tumor type has not been established yet and efficacy of mTOR inhibitor remains uncertain. These facts prompt us to investigate whether mTOR is activated in gastroenteropancreatic poorly differentiated neuroendocrine carcinomas.

In the present study, 6 (67%) of 9 poorly differentiated neuroendocrine carcinoma patients showed high expression to p-mTOR and 3 (27%) of 11 well-differentiated neuroendocrine tumor and carcinoma patients showed high expression to p-mTOR. According to the study by Yao et al. [6], the response rate of RAD001 (everolimus) against

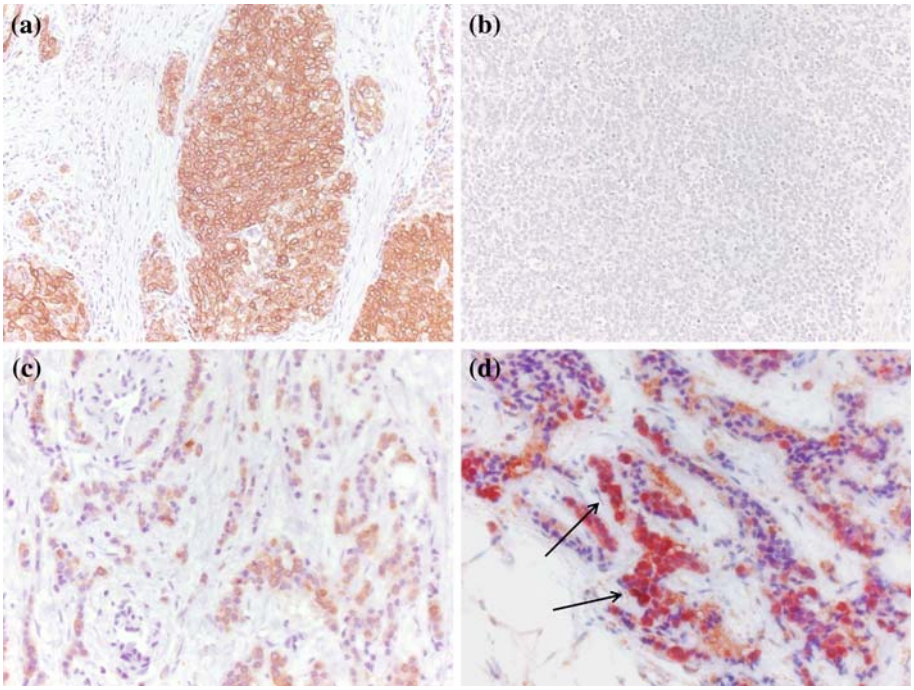

Fig. 1 Immunohistochemical staining of p-mTOR and double staining with p-Akt. **a** High expression of p-mTOR in poorly differentiated neuroendocrine carcinoma of the pancreas (case no. 7). Both cytoplasmic and membranous staining is seen. **b** No expression of p-mTOR in poorly differentiated neuroendocrine carcinoma of the pancreas (case no. 8, small-cell type). **c** Moderate expression of p-mTOR in poorly differentiated neuroendocrine carcinoma in the rectum (case no. 4). **d** Double immunohistochemical staining of p-mTOR and p-Akt. The p-mTOR positive cells are red colored by AEC and p-Akt positive cells are brown colored by DAB. Some of the tumor cells show positive to both p-mTOR and p-Akt (*arrows*)

Table 2 Immunohistochemical results of gastroenteropancreatic NET

Case	p-mTOR	p-Akt
1	++	++
2	–	–
3	–	–
4	+++	++
5	+++	++
6	++	+
7	+++	–
8	–	–
9	++	+
10	++	ND
11	–	–
12	–	–
13	++	ND
14	–	ND
15	–	ND
16	–	ND
17	–	ND
18	–	ND
19	++	+
20	–	ND

ND not done

Table 3 Correlation of p-mTOR expression with WHO classifications

WHO classification	p-mTOR (high)	p-mTOR (low)	Total
Well-differentiated neuroendocrine tumor and well-differentiated neuroendocrine carcinoma	3	8	11
Poorly differentiated neuroendocrine carcinoma	6	3	9
Total	9	11	20

$P = 0.095$ by Fisher's test

Table 4 p-mTOR expression in poorly differentiated neuroendocrine carcinoma

	p-mTOR (high)	p-mTOR (low)	Total
Small cell carcinoma	0	3	3
Large cell carcinoma	6	0	6

$P = 0.012$ by Fisher's test

low-to-intermediate grade neuroendocrine tumors was 20% which is similar to the high expression rate of p-mTOR in the present study (i.e., 27%). The higher expression rate (67%) of p-mTOR in poorly differentiated neuroendocrine carcinoma patients may suggest the efficacy of mTOR inhibitor as a therapeutic agent against this aggressive disease.

Interestingly, although all the poorly differentiated neuroendocrine carcinoma of the large-cell type showed high expression to p-mTOR, all the small cell carcinoma patients showed low expression to p-mTOR in the present study. This implies that gastroenteropancreatic small cell carcinoma patients may not benefit from mTOR inhibiting therapy. However, recent study has revealed that some small cell lung carcinoma (cell lines) responds to mTOR inhibiting therapy [10]. Therefore, whether or not mTOR inhibiting therapy is effective for small cell carcinoma needs further research. Moreover, the difference of

p-mTOR expression between large-cell type and small-cell type poorly differentiated neuroendocrine carcinomas may reflect the pathophysiological and biological difference in the tumor phenotypes [11]. However, this has to be clarified in a larger number of patients.

In the present study, not all tumor cells expressing p-mTOR co-expressed p-Akt. This means that the activation of mTOR in gastroenteropancreatic tumor patients is regulated not only by the PI3K-Akt pathway but also by other stimulants such as the nutrient status [12]. However, the difference of mTOR activation pathway would not affect the sensitivity to mTOR inhibitors. In general, mTOR inhibitors initially binds to the intracellular receptor FKBP-12 (FK 506 binding protein) and then mTOR inhibitor/FKBP-12 complex binds to mTOR leading to the dephospholylation of both p70 ribosomal S6 kinase and 4EBP1 [13]. This will result in the inhibition of mTOR signaling, translation initiation, and cell growth, leading to anti-tumor effect.

Although the sample size was limited, this is the first study to determine the expression of activated mTOR in gastroenteropancreatic neuroendocrine tumors, especially in poorly differentiated neuroendocrine carcinomas. A high expression rate of p-mTOR in poorly differentiated neuroendocrine carcinomas (large-cell type) may surely suggest the potential role of mTOR inhibitors as effective therapeutic agents for this highly malignant disease. Further research including larger number of patients and clinical trials should be considered in the future.

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