punctures in mice using Glucose pilot (Aventir Biotech), according to the procedures specified by the manufacturer. Plasma insulin levels were determined with blood samples from tail vein punctures or inferior vena cava in mice by ELISA (Morinaga Institute of Biological Science), according to the procedures specified by the manufacturer. For glucose tolerance tests, mice were fasted overnight and blood was drawn from tail vein at 0, 15, 30, 45, 60, 90, and 120 min after i.p. injection of D-glucose (2 mg/g of body weight).

Quantitative Measurement of Islet Morphology. Islet area and islet nuclei number were measured from hematoxylin and eosin-stained pancreas sections and Ki67-positive cells were counted from immunohistochemically stained pancreas sections using TissueFAXS (TissueGnostics). Chromogranin A signal intensity was also measured using TissueFAXS. α and β cells areas were quantified by the number of pixels in each immunohistochemically stained area in images taken by fluorescence microscopy (Olympus IX2-DSU).

Isolation of Rat and Mouse Primary Islets and Preparation of Primary Islet Cells. The animal experiment was reviewed and approved by the Institutional Animal Care and Use Committee for Frontier Medical Sciences, Kyoto University. For isolation of rat islets, Lewis or Wistar rats (male, aged 9–11 wk, Shimizu Laboratory Supplies) were used. Islet isolation was performed according to previously described methods (25). Briefly, through midline laparotomy, 10 mL of a type XI collagenase solution (1200 CDU/mL, C9407, Sigma-Aldrich) was infused into the common bile duct that was ligated at the hepatic side before the inflow into the duodenum. The pancreases were removed and digested in a water bath set at 37 °C for 18 min. The digested pancreases were filtered with a stainless steel sieve to separate the islets, and purified using a discontinuous gradient solution (Dextran 70, 17–0280-02, Amersham). Mouse islets were isolated from 10- to 25-wk-old male animals by collagenase digestion of the pancreas, followed by purification using a Ficoll gradient. Islets were handpicked twice. The harvested islets were cultured in RPMI or CRML-1066 medium (11530, Gibco) supplemented with a 1% antibiotic–antimycotic solution (15240-062, Gibco) and 10% (vol/vol) FBS (12103-78P, JRH) in an incubator set at 5% (vol/vol) CO_2, 37 °C.

Primary islet cells were prepared by digesting the islets with Accutase for 15 min at 37 °C. Islet cells were washed with RPMI before use in experiments.

Immunohistochemistry. Immunohistochemistry (IHC) was performed basically according to the manufacturer's instructions. In brief, after deparaffinization, tissues sections underwent antigen retrieval by autoclaving slides for 5 min in 10 mM citrate buffer (pH 6.0). For fluorescent immunohistochemical staining of insulin, glucagon, and Glut2, nonspecific interactions were blocked for 30 min using a 5% (vol/vol) goat serum solution. The primary antibodies were: guinea pig anti-insulin polyclonal antibody (Abcam) diluted 1:400, mouse anti-glucagon monoclonal antibody (Sigma-Aldrich) diluted 1:750 and rabbit anti-Glut2 polyclonal antibody (Alpha Diagnostic) diluted 1:750 with Signal Enhancer HIKARI (Nacalai Tesque). These were applied to the slides and incubated overnight at 4 °C. As secondary antibodies, Alexa Fluor 488 goat anti-mouse IgG antibody (Invitrogen) diluted 1:500, Alexa Fluor 488 goat anti-rabbit IgG antibody (Invitrogen) diluted 1:500 and Alexa Fluor 546 goat anti-guinea pig IgG antibody (Invitrogen) diluted 1:1,000 with PBST-BSA were applied to the slides and incubated 3 h at room temperature. To detect Ki67- and Chromogranin A-positive cells, sections were pretreated with 0.3% H_2O_2 for inactivation of endogenous peroxidase. The primary antibody, rat anti-Ki67 monoclonal antibody (DakoCytomation) diluted 1:200, or rabbit anti-Chromogranin A polyclonal antibody (Thermo Scientific) diluted 1:200 with Signal Enhancer HIKARI were applied to the slides and incubated overnight at 4 °C. As secondary antibodies, Histofine Simple Stain MAX PO anti-rat IgG antibody (Nichirei Bioscience) or biotinylated anti-rabbit IgG antibody (VECTOR Laboratories) was used. We used 3,3′-diaminobenzidine tetrahydrochloride (DAB; Muto Pure Chemicals) as the substrate chromogen. The sections were counter stained with hematoxylin.

STZ-Induced Diabetes. Eleven- to 22-wk-old wild-type and *PHLDA3* knockout male mice were injected i.p. with 50 mg/kg streptozotocin daily for 5 consecutive days (Sigma-Aldrich) to produce β cell injury. On days 92, 93, 99, and 100, animals were killed.

Statistical Analysis. Data were calculated and shown as mean ± SD (for Figs. 4, 5 and *SI Appendix*, Fig. S3) or as mean ± SEM (Figs. 6–8 and *SI Appendix*, Figs. S7, S9, and S10*A*). Comparisons between the samples were performed by Student *t* test. Survival data were analyzed using XLStat software (version 2013.4.05; Addinsoft), and Kaplan–Meyer plots were drawn. Wilcoxon test was performed to assess the statistical significance of the difference between the survival curves. In Fisher's exact test, *P* values were obtained by using two tails. Statistical significance was defined as $P < 0.05$.

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Does the WHO 2010 classification of pancreatic neuroendocrine neoplasms accurately characterize pancreatic neuroendocrine carcinomas?

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Abstract
Background The WHO classified pancreatic neuroendocrine neoplasms in 2010 as G1, G2, and neuroendocrine carcinoma (NEC), according to the Ki67 labeling index (LI). However, the clinical behavior of NEC is still not fully studied. We aimed to clarify the clinicopathological and molecular characteristics of NECs.
Methods We retrospectively evaluated the clinicopathological characteristics, *KRAS* mutation status, treatment response, and the overall survival of eleven pNEC patients diagnosed between 2001 and 2014 according to the WHO 2010. We subclassified WHO-NECs into well-differentiated NEC (WDNEC) and poorly differentiated NEC (PDNEC). The latter was further subdivided into large-cell and small-cell subtypes.
Results The median Ki67 LI was 69.1 % (range 40–95 %). Eleven WHO-NECs were subclassified into 4 WDNECs and 7 PDNECs. The latter was further separated into 3 large-cell and 4 small-cell subtypes. Comparisons of WDNEC vs. PDNEC revealed the following traits: hypervascularity on CT, 50 % (2/4) vs. 0 % (0/7) ($P = 0.109$); median Ki67 LI, 46.3 % (40–53 %) vs. 85 % (54–95 %) ($P = 0.001$); Rb immunopositivity, 100 % (4/4) vs. 14 % (1/7) ($P = 0.015$); *KRAS* mutations, 0 % (0/4) vs. 86 % (6/7) ($P = 0.015$); response rates to platinum-based chemotherapy, 0 % (0/2) vs. 100 % (4/4) ($P = 0.067$), and median survival, 227 vs. 186 days ($P = 0.227$).
Conclusions The WHO-NEC category may be composed of heterogeneous disease entities, namely WDNEC and PDNEC. These subgroups tended to exhibit differing profiles of Ki67 LI, Rb immunopositivity and *KRAS* mutation, and distinct response to chemotherapy. Further studies for the reevaluation of the current WHO 2010 classification are warranted.

Keywords Neuroendocrine carcinoma · Ki67 labeling index · *KRAS* mutation · WHO classification

Abbreviations

NEN	Neuroendocrine neoplasm
WHO	World Health Organization

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NET	Neuroendocrine tumor
NEC	Neuroendocrine carcinoma
EUS-FNA	Endoscopic ultrasound-guided fine needle aspiration
ENETS	European Neuroendocrine Tumor Society
IHC	Immunohistochemistry
PCR	Polymerase chain reaction
SD	Standard deviation
LCNEC	Large-cell NEC
SCNEC	Small cell-NEC
PDAC	Pancreatic ductal adenocarcinoma

Introduction

Ki67 is a powerful prognostic marker of pancreatic neuroendocrine neoplasms (pNENs) [1] and, accordingly, the remarkable revision was made from the former 2000 World Health Organization (WHO) classification system to the current WHO 2010 terminology system, in which mitotic count and/or Ki67 labeling index (LI) were adopted as the pivotal indicator of stratification [2]. NENs are now to be categorized into neuroendocrine tumor (NET)-G1, NET-G2, and neuroendocrine carcinoma (NEC). Whereas NETs-G1/G2 are invariably composed of tumor cells with well-differentiated morphology, NECs usually have poorly differentiated histology with Ki67 LI > 20 % [2, 3]. Accordingly, all NENs with Ki67 LI > 20 % are defined as NEC. Clinically, these tumors are treated with the same platinum-based chemotherapy regimens as small-cell lung cancers [4–6]. However, some reports have recently indicated that a proportion of well-differentiated NENs might have proliferative rates above the threshold for NET-G2 [7, 8]. In addition, the Nordic NEC study reported that patients with a Ki67 <55 % had low responses to platinum-based chemotherapy [9]. We suppose that the current NEC category, as defined by the WHO 2010 classification (WHO-NEC), includes two groups that differ in clinical behaviors as well as pathological characteristics. Information about the clinicopathological features of WHO-NEC group is scant [7–10]. Therefore, we aimed to further characterize the WHO-NEC group in terms of pathological findings, molecular characteristics, and clinical behaviors.

Patients and methods

Patients

We retrospectively retrieved all of the pNENs diagnosed between January 2001 and March 2014 from our hospital database. All patients were recategorized as NET-G1, NET-G2, or NEC according to the WHO 2010 classification. Specimens for histological examination were obtained from preoperative endoscopic ultrasound-guided fine needle aspiration (EUS-FNA), biopsy, and/or surgical resection. All patients diagnosed with small-cell carcinoma were subsequently assessed by contrast enhanced (CE) chest MDCT to exclude the possibility of metastasis from a primary lung cancer [11]. This study was approved by our institutional review board.

Diagnostic and prognostic characterization

The following features were recorded for all patients: age, gender, symptoms, hormonal syndromes, primary and metastatic locations, European Neuroendocrine Tumor Society (ENETS) TNM stage [12], and CE-MDCT features such as anatomical location, tumor size, and contrast enhancement. We recorded the details of all treatments administered to the patients, particularly platinum-based chemotherapy [4, 5, 13].

Endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) and sample preparation

EUS-FNA procedures were performed using a convex linear-array echoendoscope (GF-UGT240 or GF-UCT260; Olympus Optical Co Ltd, Tokyo, Japan) paired with an ultrasound machine (SSD5500 or Prosound α10; Aloka, Tokyo, Japan). We used 22-gauge needles (NA-11J-KBor NA-200H-8022; Olympus Medical System Corp. Ltd., Tokyo, Japan or EchoTip-Ultra Needle; Cook Endoscopy Inc., Winston Salem, N.C., USA or Expect; Boston Scientific Japan, Tokyo, Japan).

Aspirated materials were divided for cytopathological evaluation, cell-block preparation, and *KRAS* mutation analysis. In all patients, specimen adequacy was evaluated on-site by Diff Quick staining (Diff-Quik; Kokusai Shiyaku, Kobe, Japan) by a cytopathologist or cytotechnologist. Cell-blocks were prepared after the fresh specimens were immediately fixed in 10 % formalin and embedded in paraffin. Sliced sections then were stained by hematoxylin and eosin, as well as by immunohistochemical staining (IHC) [14].

Histological evaluation

We defined tumors as NEC that showed diffuse expression of neuroendocrine markers and Ki67 LI of more than 20 %. In accordance with the 2010 WHO classification, tumors characterized by high-grade cytological atypia, apparent pleomorphism, extensive necrosis, and prominent mitotic activity were categorized into poorly differentiated NEC

(PDNEC). Of PDNECs, tumors characterized by diffuse growth of highly atypical cells with small-sized to medium-sized nuclei, finely granular chromatin, and inconspicuous nucleoli, were categorized as small-cell NEC (SCNEC). Carcinomas with large nuclei, coarse chromatin and well-visible nucleoli with nested proliferation were categorized as large-cell NEC (LCNEC). Furthermore, we attempted to extract those tumors whose cytological features were blander than that of PDNEC and rather similar to NET-G2; that is, tumors composed predominantly of cells with low nucleocytoplasmic ratio and small-sized to medium-sized, ovoid nuclei, growing with minimal pleomorphism, and lacking extensive necrosis. We designated these tumors as 'well differentiated NEC (WDNEC)', and separated them from SCNECs and LCNECs. All slides were reviewed and reclassified by the same pathologist (WH).

Immunohistochemistry and Ki67 labeling index

IHC was performed using monoclonal antibodies for chromogranin A (clone SP12, rabbit, 1:200, Neo Markers), synaptophysin (clone SP11, rabbit, 1:100, Neo Markers, Fremont, CA, USA), Ki67 (clone SP6, rabbit, 1:200; Neo Markers), and Rb (clone 3H9, mouse, 1:300; MBL).

The measurement of Ki67 LI was performed under the assistance of digital pathology technology. Briefly, slides were digitally scanned using a Scan Scope XT (Aperio Technologies, Vista, CA, USA). All sections were reviewed to exclude portions with extensive desmoplasia, necrosis and regions with bleeding. The ultimate Ki67 LI was determined as the highest value found in each specimen using the IHC Nuclear Image Analysis tool (Aperio Technologies, Vista, CA, USA) and was similarly measured and determined in cell-block sections of EUS-FNA specimens as described previously [15].

The prominent concern about EUS-FNA is whether WHO classification (grading) is possible with the biopsy specimens. We previously reported a study [15] about a comparison of grades of pNENs between resected and EUS-FNA specimens by Ki67 immunostaining. The concordance rate rose to 90 % when EUS-FNA samples contained more than 2000 neoplastic cells. In accordance with our previous study, we defined the cases whose neoplastic cells were insufficient for grading (less than 2000 cells) as tumors of 'uncertain' grade.

Analysis of KRAS mutation

Genetic analysis was performed on either the fresh specimens or formalin-fixed paraffin-embedded sections. After nucleic acids were extracted and amplified by polymerase chain reaction, gene mutations were analyzed by ABI PRISM 310 Genetic Analyzer (Applied Biosystems) or the Cycleave PCR assay (Takara Co., Ltd); the detail of which was described previously [16, 17].

Statistical analysis

Statistical analysis was performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) software and P values <0.05 were considered statistically significant. Categorical variables are expressed as absolute (n) and relative (%) frequencies and were compared using the Chi squared test or Fisher's exact test. Survival was analyzed using the Kaplan–Meier method with the log-rank test.

Results

Ninety-five patients were diagnosed with pNEN at our hospital during the study period. As to grading of pNENs, the WHO classification 2010 suggests two parameters (mitotic count and Ki67 LI) to evaluate the proliferative activity of tumors. We performed grading of pNENs by measuring Ki67 LI and did not employ the mitotic count method, because our study consisted mostly of tumors diagnosed by FNA specimens, which were too small an amount to secure 50 microscopic fields necessary for the calculation of mitotic count. The pNENs were reclassified into uncertain for Ki67 LI ($n = 8$), NET-G1 ($n = 55$), NET-G2 ($n = 21$), and WHO-NEC ($n = 11$) in accordance with the WHO 2010 classification. The 11 cases of WHO-NEC were the subject of analysis in this study (Fig. 1).

Basic demographic and clinical features of patients with WHO-NEC (Tables 1, 2)

Ten (91 %) of 11 patients were symptomatic, mainly with abdominal pain. The median tumor size was 35 mm (range 20–55 mm). Tumors were located in the head, body, and tail of the pancreas in 2, 5, and 4 patients, respectively. Eight (72 %) patients had liver metastasis at the time of diagnosis, two were treated with surgery (ENETS stageIIb and IIIb) and six who received platinum-based chemotherapy (3 cases were cisplatin + irinotecan and 3 cases were cisplatin + etoposide) had a response rate of 67 %. In the remaining 2 patients, one patient received Gemcitabine (case 3) and another patient received Everolimus because we defined it as WDNEC (case 9). The overall median survival was 314 days (range 60–1202 days).

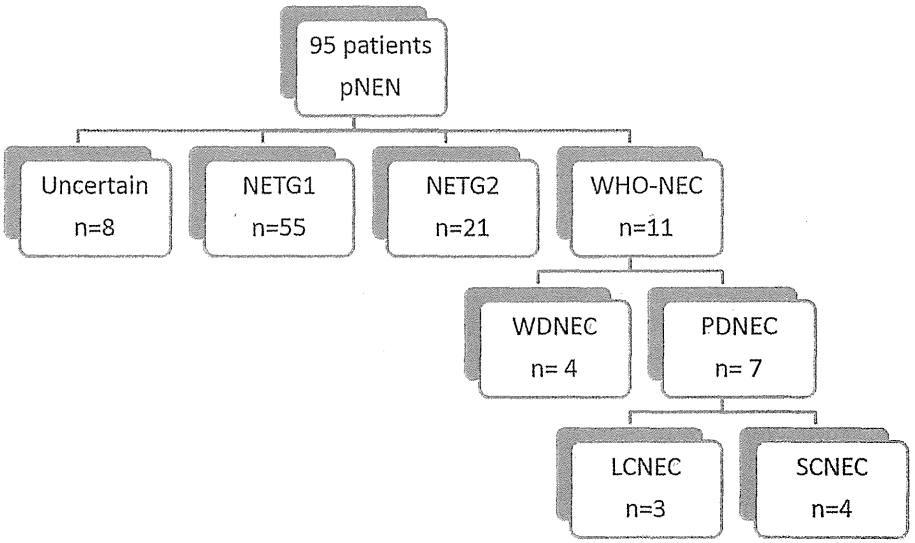

Fig. 1 Algorithm for patient selection from pNEN. *NEN* neuroendocrine neoplasm, *NET* neuroendocrine tumor, *LCNEC* large cell NEC, *SCNEC* small cell NEC, *WDNEC* well-differentiated neuroendocrine carcinoma, *PDNEC* poorly-differentiated neuroendocrine carcinoma

Table 1 Patient characteristics ($n = 11$)

Gender	
Male/female	6/5
Age	
Median (range)	59 years (28–74)
Symptom	
Yes (%)	91 % (abdominal pain)
Site of pancreas tumor	
Head/body/tail	2/5/4
Tumor size	
Median (range)	35 mm (20–55)
Metastasis	
Yes (%)	72 % (liver metastasis)
Treatment	
Operation/chemotherapy/BSC	2/8/1

Imaging features of WHO-NEC on CE-MDCT (Fig. 2; Supplementary Table)

Assessment by CE-MDCT revealed that 9 (82 %) of 11 WHO-NEC in the pancreas were hypovascular. Eight of these tumors had metastasized to the liver, where 7 (88 %) of them were also hypovascular, like the primary tumor (Fig. 2). Before biopsy confirmation, NEN were suspected in only two patients, and the imaging features in the remaining 9 (82 %), suggested pancreatic ductal adenocarcinoma (PDAC). The main pancreatic duct was dilated in 4 (57 %) of 7 patients with tumors located in the head and body of the pancreas.

Pathological and molecular characteristics of WHO-NEC (Fig. 3, Supplementary Figure; Tables 2, 3)

A total of 11 WHO-NEC cases were submitted to the pathological and molecular analysis. No ductal carcinoma components were noted. All cases showed diffuse and strong immunoreactivity for neuroendocrine markers except 1 case, in which only synaptophysin was positive. In total, chromogranin A was expressed in 91 % and synaptophysin was expressed in 100 % of cases. The median Ki67 LI was 69.1 % (range 40–95 %). Nuclear expression of Rb protein was retained in 5 (45 %) tumors. *KRAS* mutations were detected in 6 (55 %) tumors. Seven (64 %) and 4 (36 %) of 11 tumors were categorized as PDNEC (4 SCNECs and 3 LCNECs) and WDNEC, respectively, according to their morphologic characteristics that we mentioned in the "Patients and methods" (Fig. 3, Supplementary Figure).

Clinicopathological comparison of well-differentiated and poorly differentiated NEC (Table 4)

The clinicopathological comparison between the WDNEC and PDNEC groups revealed that they were clinically and molecularly different in several aspects as follows: hypervascularity in MDCT images, 50 % (2/4) vs. 0 % (0/7), $P = 0.109$; median Ki67 LI, 46 % (range 40–53 %) vs. 85 % (range 54–95 %), $P = 0.001$; nuclear expression of Rb, 100 % (4/4) vs. 14 % (1/7), $P = 0.015$; *KRAS* mutations, 0 % (0/4) vs. 86 % (6/7), $P = 0.015$; response rates to platinum-based chemotherapy, 0 % (0/2) vs. 100 % (4/4) $P = 0.067$; and median survival, 227 vs. 186 days, $P = 0.227$.

Table 2 Clinical, pathological features, treatment and response for chemotherapy of WHO-NEC patients

Case	Age/ sex	Location	Size (mm)	ENETS stage	Tissue sampling	Histology	Ki67 LI (%)	CGA	Synaptophysin	Rb	*KRAS*	Treatment	Response for platinum-based regimen
1	30, M	Body	45	IIb	Biopsy and surgical resection	WDNEC	40	Positive	Positive	Positive	WT	Operation	ND
2	59, F	Body	30	IIIb	Biopsy and surgical resection	PDNEC (small cell)	80	Positive	Positive	Positive	MT	Operation	ND
3	49, F	Body	35	IV	Biopsy	PDNEC (large cell)	85	Positive	Positive	Negative	MT	CT (Gemcitabine)	ND
4	68, F	Tail	36	IV	Biopsy	WDNEC	48	Positive	Positive	Positive	WT	CT (IP)	PD
5	63, F	Body	33	IV	Biopsy	PDNEC (large cell)	54	Positive	Positive	Negative	MT	CT (IP)	PR
6	61, M	Body	45	IV	Biopsy	PDNEC (large cell)	90	Positive	Positive	Negative	MT	CT (EP)	PR
7	74, M	Head	20	IV	Biopsy	PDNEC (small cell)	90	Positive	Positive	Negative	WT	BSC	ND
8	37, M	Head	20	IV	Biopsy	PDNEC (small cell)	80	Positive	Positive	Negative	MT	CT (EP)	PR
9	50, F	Tail	35	IV	Biopsy	WDNEC	45	Negative	Positive	Positive	WT	CT (Everolimus)	ND
10	55, M	Tail	30	IV	Biopsy	WDNEC	53	Positive	Positive	Positive	WT	CT (EP)	PD
11	66, M	Tail	70	IV	Biopsy	PDNEC (small cell)	95	Positive	Positive	Negative	MT	CT (IP)	PR

CGA chromogranin A, *WT* wild type, *MT* mutant, *CT* chemotherapy, *IP* cisplatin + irinotecan, *EP* cisplatin + etoposide, *BSC* best supportive care, *ND* not done, *PD* progressive disease, *PR* partial response

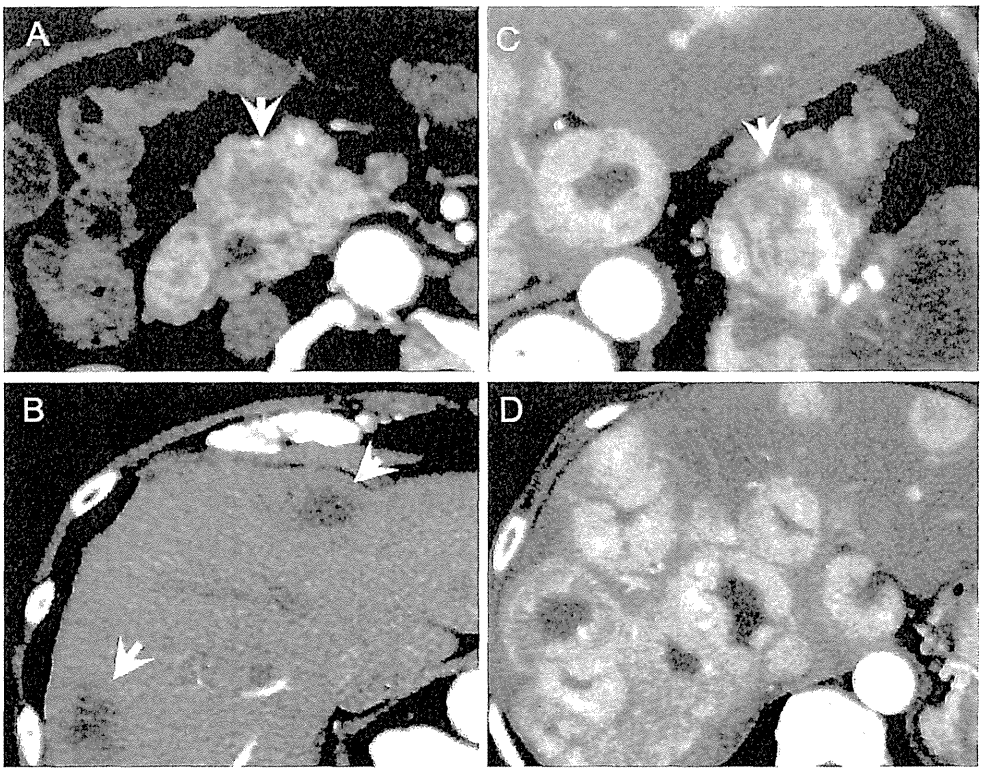

Fig. 2 Computed tomography findings of respective pNECs. **a**, **b** Hypovascular lesions both primary pancreas head site and multiple liver lesions (SCNEC case). **c**, **d** Hypervascular lesions both primary pancreas head site and multiple liver lesions (WDNEC case)

Discussion

When the WHO 2010 classification was applied to our patients with NENs of the pancreas, we found that 36 % of the high-grade category included tumors with well differentiated morphology. This critical finding has an impact on the treatment strategies, particularly the platinum-based chemotherapy which should be originally administered for only PDNEC.

Our findings suggested that WDNECs differ from PDNECs and are rather more closely related to NETs-G2 in terms of clinicopathological and molecular characteristics. Firstly, MDCT consistently showed hypervascularity in WDNEC, but not in PDNEC. Some reports indicated that tumor vascularity correlated with the proliferation index and/or WHO classification [18, 19]. Our findings indicated that only 18 % of WHO-NEC cases were suspected of pNEN according to imaging findings before EUS-FNA, with most being considered PDAC or pancreatic adeno-squamous carcinoma. That is, a significant proportion (82 %) of NECs could not be correctly diagnosed by imaging, especially the PDNEC type.

Histologically, WDNECs shared more morphological traits with NETs-G2 than PDNECs, allowing us to presume that WDNECs correspond to well-differentiated NETs with high proliferative activity. The Ki67 LI tended to be lower in WDNEC than in PDNEC. Notably, *KRAS* and *Rb* genes are promising molecular markers with which to distinguish these types of tumors. The result that *KRAS* mutations were not found in WDNECs supports the notion that this category lies in close proximity to NET-G2, as no pancreatic NETs-G1/G2 have been reported to possess *KRAS* mutations, whereas PDNECs have been shown to harbor *KRAS* mutations [10, 16, 20]. Loss of expression of Rb was found in 86 % of PDNEC cases, whereas all of the WDNEC cases retained its expression. Aberration of the Rb/p16 pathway has been reported to be frequently involved in PDNECs of the pancreas, gallbladder, and ampulla, but not in pancreatic well-differentiated NETs [10, 20–22]. Concerning pancreatic NEN, Yachida et al. [10] conducted immunohistochemical and genetic analyses of several oncogenes and tumor suppressor genes including *KRAS* and *Rb*, and revealed that the aberrations of both genes were common in PDNECs but none in NETs-G1/G2. Their conclusion that PDNECs were molecularly distinct from well-differentiated NETs is in keeping with our findings. Taken together, the difference between WDNEC and PDNEC appears to be clinically, histologically, and molecularly significant, and we consider that WDNECs are more likely to be in the category of well-differentiated NET rather than NEC, thus, favoring the designation, namely "NET-G3".

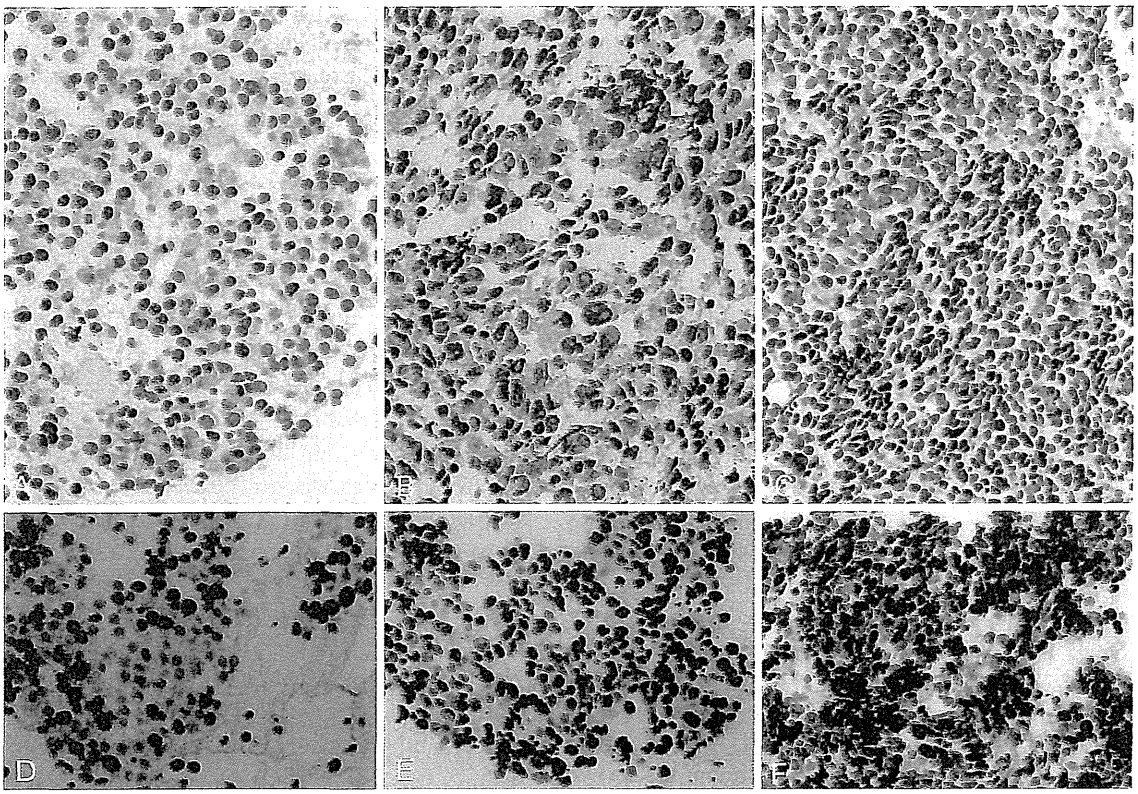

Fig. 3 Histologic features of NECs of the pancreas [H&E stain (**a–c**), and Ki67 (**d–f**), respectively]. The *left column* (**a**, **d**) is a case of WDNEC, the *middle column* (**b**, **e**) is of LCNEC, and the *right column* (**c**, **f**) is of SCNEC. Morphology of WDNECs shows a close similarity to that of NET-G1/G2, characterized by monomorphic growth of tumor cells with highly preserved endocrine cell features. Although LCNECs have features of endocrine cells as well, they are distinguished from WDNECs by increased nuclear atypia, cellular pleomorphism, and the frequent presence of tumor necrosis. SCNECs are composed of small cells with dense chromatin, scarce cytoplasm, and remarkable mitotic activity. These are reminiscent of small cell carcinomas of the lung

Table 3 Pathological and molecular characteristics of WHO-NEC

Ki67 labeling index	
Median (range)	69.1 % (40–95 %)
Morphology	
WDNEC/PDNEC	4/7
Subtypes of PDNEC	
Large-cell type/small-cell type	3/4
Rb immunopositivity	45 % (5/11)
KRAS mutation	54 % (6/11)

WDNEC well-differentiated NEC, *PDNEC* poorly differentiated NEC

Table 4 Clinicopathological comparison of WDNEC and PDNEC

	WDNEC ($n = 4$)	PDNEC ($n = 7$)
Vascularity in pancreas tumor		
Yes (%)	50 % (2/4)	0 % (0/7)
Ki67 labeling index		
Median (range)	46.3 % (40–53 %)	85 % (54–95 %)
Rb immunopositivity	100 % (4/4)	14 % (1/7)
KRAS mutation	0 % (0/4)	86 % (6/7)
Response rate of platinum-based regimen	0 % (0/2)	100 % (4/4)
Prognosis		
Median	227 days	186 days

WDNEC well-differentiated NEC, *PDNEC* poorly differentiated NEC

Our study showed that both WDNEC and PDNEC patients harbored unfavorable outcome (median overall survival of 227 days and 186 days, respectively), which is in stark contrast to NET-G2 patients whose median overall survival is reportedly 162 months [1]. Although WDNEC and PDNEC shared aggressiveness clinically and pathologically, the efficacy of the treatment between them tended to be different; all WDNEC cases did not exhibit response to the platinum-based chemotherapy while all of the PDNEC cases did. The Nordic NEC study [9] found that WHO-NEC with Ki67 LI > 55 % responded to platinum-based chemotherapy, whereas those with Ki67 LI < 55 % did not. Although the Nordic NEC study mainly focused on the treatment and prognostic aspects, there was no detailed description of the pathologic

characteristics of the cases. We suppose that some of their WHO-NEC included WDNEC as defined herein. Based on the results of the Nordic NEC study, the NCCN guidelines noted in footnotes that "intermediate Ki67 levels in the 20–50 % range may not respond well to platinum/etoposide as patients with small cell histology or extremely high Ki67 and so, a clinical judgment should be used". When NEN is diagnosed as WHO-NEC, clinically the toxic platinum-based chemotherapy is usually administered as a first-line regimen. However, a recent case report showed a good response of high-grade NET to molecular targeted therapy with agents such as Everolimus [23]. In fact, one patient who was diagnosed with WDNEC and received Everolimus obtained partial response. The current WHO 2010 classification might be flawed in terms of the management of patients with NEC and the classification scheme for NECs should be revised as the clinical, pathological, and molecular characteristics of this high-grade NEN become more fully clarified.

In regard to IHC, chromogranin A was expressed in 91 % of WHO-NEC cases, and synaptophysin was expressed in 100 %. In a similar fashion, previous articles reported that chromogranin A was expressed in 81–94 %, and synaptophysin was expressed in 88–96 % [7–9]. Taken together, stainability of chromogranin A and synaptophysin is high not only in WDNEC but also in PDNEC.

In our institute, we perform EUS-FNA for the diagnosis of pancreatic tumors on a routine basis, and have been reported its usefulness so far [11, 14–16, 24]. The diagnostic accuracy of overall pancreatic tumors was 91.8 % (918/996) [14]. We previously detected *KRAS* mutations in 87 % (266/307) of EUS-FNA specimens from pancreatic masses in patients with PDAC [24] and none among 25 well-differentiated endocrine tumors [16]. Jiao et al. [20] also reported the absence of *KRAS* mutations in NET-G1/G2.

To the best of our knowledge, this is the first study which examined the clinicopathological characteristics of pNECs, with an emphasis on the difference between WDNEC and PDNEC. However, some limitations should be addressed. The retrospective design hindered precise analysis of all required data, imposed potential selection bias, and the patient cohort was small due to the natural rarity of pNECs that account for <1 % of all pancreatic carcinomas, and 2–7.5 % of all pNEN [2, 25]. Intratumoral heterogeneity is another important consideration. In our 11 cases of NEC, we did not note any adenocarcinoma component histologically nor immunohistochemically. Also, the result of the high frequency of Rb aberration in our series minimizes the possibility of a hidden presence of concomitant adenocarcinomas, as Rb aberration has been reported to be a rare event in PDACs (5–6 %) [26, 27]. Although the above observations do not fully rule out the possibility that some of the cases might contain an accompanying adenocarcinoma, this may be a relatively uncommon occurrence given the low frequency of an associated ductal adenocarcinoma in PDNECs reported by Basturk et al. [8] (6/44, 14 %). Finally, we address the feasibility of grading for pNENs diagnosed by FNA specimens, which constituted most of our series. Past studies of ours and of others claimed that grading by Ki67 LI can be applicable to FNA specimens by showing high concordance between the grade given by the FNA specimens and that by the corresponding resected specimens (concordance rate 78–90 %) [15, 28–31]. Indeed, downgrading or upgrading between G1 and G2 occurred in a small proportion of cases, but there was no tumor observed among the 5 studies that was graded as G3 by EUS-FNA and was downgraded to G2 by surgical resection. This observation, as well as the poor outcome of the current study, indicates that the admixture of 'overestimated' NETs-G2 in our cohort seemed unlikely to happen.

In conclusion, we identified a significant number of "WDNEC" cases among pNECs that were defined by the current WHO classification system. The clinicopathological and molecular analyses suggested that WDNEC is distinct from PDNEC. Though the number of cases we analyzed was limited, we believe that our scheme of subcategorizing pancreatic NEC showed promise. Further larger-scale studies are warranted to validate our stratification of WHO-NECs, which will facilitate a more personalized treatment of the patients with this rare malignant neoplasm.

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Conflict of interest The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Epidemiological trends of pancreatic and gastrointestinal neuroendocrine tumors in Japan: a nationwide survey analysis

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Abstract
Background Although neuroendocrine tumors (NETs) are rare, the number of patients with NET is increasing. However, in Japan, there have been no epidemiological studies on NET since 2005; thus, the prevalence of NET remains unknown.
Methods We reported the epidemiology of gastroenteropancreatic neuroendocrine tumors (GEP-NETs) [pancreatic neuroendocrine tumors (PNETs) and gastrointestinal neuroendocrine tumors (GI-NETs)] in Japan in 2005. Here, we conducted the second nationwide survey on patients with GEP-NETs who received treatment in 2010.
Results A total of 3,379 patients received treatment for PNETs in 2010, representing a 1.2-fold increase in the number of patients from 2005 to 2010. The prevalence was estimated to be 2.69/100,000, with an annual onset incidence of 1.27/100,000 in 2010. Non-functioning tumor (NF)-PNETs comprised 65.5 % of cases followed by insulinoma (20.9 %) and gastrinoma (8.2 %). Interestingly, the number of patients with NF-PNETs increased ~1.8 fold since 2005. A total of 19.9 % of patients exhibited distant metastasis at initial diagnosis; 4.3 % had complications with multiple endocrine neoplasia type 1 (MEN-1), and only 4.0 % had NF-PNETs associated with MEN-1. Meanwhile, an estimated 8,088 patients received treatment for GI-NETs, representing a ~1.8-fold increase since 2005. The prevalence was estimated to be 6.42/100,000, with an annual onset incidence of 3.51/100,000. The locations of GI-NETs varied: foregut, 26.1 %; midgut, 3.6 %; and hindgut, 70.3 %. Distant metastasis and complications with MEN-1 were observed in 6.0 and 0.42 % at initial diagnosis, respectively. The frequency of carcinoid syndrome in patients with GI-NETs was 3.2 %.
Conclusion We clarified the epidemiological changes in GEP-NETs from 2005 to 2010 in Japan.

Keywords Neuroendocrine tumor · Pancreatic neuroendocrine tumor · Gastrointestinal neuroendocrine tumor · Nation-wide survey · Epidemiology

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Introduction

Neuroendocrine tumors (NETs) are generally considered rare tumors that progress slowly [1]. However, according to the surveillance, epidemiology, and end results (SEER) study, a US epidemiological database, the number of patients has been increasing; the incidence rate of the disease increased fivefold from 1.09 per 100,000 people in 1973 to 5.25 per 100,000 people in 2004 [2]. Although the reasons for this increase are unclear, the recognition of the disease and improved diagnostic technology may be partially responsible. Thus, continued accumulation and examination of data regarding the trend of the actual number of patients is necessary [3, 4].

However, in Japan, the prevalence of gastroenteropancreatic NETs (GEP-NETs) [pancreatic endocrine tumors (PNETs) and gastrointestinal neuroendocrine tumors (GI-NETs)] is unclear. Consequently, a nationwide epidemiological survey of patients with GEP-NET who received treatment from January 1 to December 31, 2005 was conducted in [5]; thus, the difference in the prevalence of the disease between Japan and Western nations gradually became clear. The large differences in GEP-NETs between Japan and Western nations are primarily due to differences in the presence of multiple endocrine neoplasia type 1 (MEN-1) in NF-PETs as well as the location, symptomatic status, and prevalence of malignancy in GI-NETs [5]. The present study reports the second nationwide survey on patients with GEP-NETs who received treatment in 2010. Furthermore, the epidemiological changes in these patients from 2005 to 2010 were examined.

Methods

We conducted the second nationwide survey to examine the epidemiology of GEP-NETs in Japan. The subjects were patients with GEP-NETs including PNETs and GI-NETs who received treatment from January 1 to December 31, 2010. Subjects were collected using a nationwide stratified random sampling method similar to that used in the first survey [5]. In brief, the departments of gastroenterology, gastroenterological surgery, endocrinology, and metabolic medicine of each hospital were listed, and stratified random sampling was used to select departments for the survey. The sampling rates for the stratum of general hospitals with <100, 100–199, 200–299, 300–399, 400–499, and ≥500 beds and university hospitals were 5, 10, 20, 40, 80, 100, and 100 %, respectively. To increase the efficiency of this survey, we added some relevant departments in which many patients with GEP-NETs were expected to be treated; they were considered a special stratum and were all selected. A questionnaire was directly mailed to the heads of the 6,339 randomly selected departments at the abovementioned sampling rates. Returned questionnaires providing information about 3,366 patients including 1,273 patients with PNETs and 2,093 with GI-NETs were collected. A response rate was 20.2 %. The diagnosis of GEP-NETs was classified according to the WHO 2010 criteria [6]. However, mixed adenoneuroendocrine carcinoma (MANEC) and hyperplastic and preneoplastic lesions were excluded. Regarding PNETs, patients with clinical symptoms and elevated plasma hormone levels were diagnosed as having a functioning PNET. On the other hand, patients without clinical symptoms and with no elevation of plasma hormone levels were diagnosed as having a nonfunctioning tumor (NF-PNET) regardless of whether the hormone production was evaluated by immunohistochemistry or mRNA detection in the tumor tissue.

Results

Epidemiology of PNETs in Japan

Epidemiology (Table 1)

The data collected from the present survey showed the estimated total number of patients treated for PNETs in the year 2010 was 3,379 [95 % confidence interval (CI) 3,173–3,580] and the overall prevalence was 2.69 per 100,000 people (95 % CI 2.29–3.08). This represents an approximately 1.2-fold increase since 2005. The total number of patients treated for functioning tumors was estimated to be 1,105 (95 % CI 868–1,342), and the overall prevalence was 0.88 per 100,000 people (95 % CI 0.65–1.05). On the other hand, the total number of patients treated for non-functioning tumors was estimated to be 2,274 (95 % CI 1,759–2,789), and the overall prevalence was 1.81 per 100,000 people (95 % CI 1.51–2.11). There were more patients with functioning PNETs than NF-PNETs in 2005, while the opposite trend was observed in 2010. The incidence rates of PNETs, functioning tumors, and NF-PETs in 2010 were estimated to be 1.27 per 100,000 people (95 % CI 1.08–1.46), 0.41 per 100,000 people (95 % CI 0.32–0.48), and 0.87 per 100,000 people (95 % CI 0.72–1.01), respectively. The number of new-onset functioning PNETs in 2010 was similar to that in 2005; however, the number of new-onset NF-PNETs in 2010 was approximately 1.7-fold greater than that in 2005.

Distribution of PNETs in Japan in 2010 (Table 2)

NF-PNETs were the most common PNETs in Japan in 2010, comprising 65.5 % of all PNETs. Meanwhile, functioning tumors comprised 34.5 % of PNETs. The most

Table 1 The trends of epidemiology of pancreatic neuroendocrine tumors (PNETs) from 2005 to 2010 in Japan

	2005*	2010
Total number of patients treated for PNET	2,845 (95 % CI 2,455–3,507)	3,379 (95 % CI 3,173–3,580)
Functioning tumors	1,627 (95 % CI 1,404–2,005)	1,105 (95 % CI 868–1,342)
Non-functioning tumors	1,218 (95 % CI 1,053–1,453)	2,274 (95 % CI 1,759–2,789)
Overall prevalence of PNETs (per 100,000 population)	2.23 (95 % CI 1.93–2.76)	2.69 (95 % CI 2.29–3.08)
Functioning tumors	1.27 (95 % CI 1.10–1.57)	0.88 (95 % CI 0.65–1.05)
Non-functioning tumors	0.95 (95 % CI 0.82–1.17)	1.81 (95 % CI 1.51–2.11)
Incidence rate of PNETs (per 100,000 population)	1.01 (95 % CI 0.88–1.25)	1.27 (95 % CI 1.08–1.46)
Functioning tumors	0.5 (95 % CI 0.44–0.62)	0.41 (95 % CI 0.32–0.48)
Non-functioning tumors	0.51 (95 % CI 0.88–1.25)	0.87 (95 % CI 0.72–1.01)

*Data modified from reference [5]

95 % CI 95 % confidence interval

Table 2 Distribution of pancreatic neuroendocrine tumors (PNETs) in 2010

	Number of patients	Percentage (%)
Functioning PNETs	439/1,273	34.5
Insulinoma	266/1,273	20.9
Gastrinoma	104/1,273	8.2
Glucagonoma	42/1,273	3.2
VIPoma	8/1,273	0.6
Somatostatinoma	4/1,273	0.3
Others	17/1273	1.3
Non-functioning PNETs	834/1,273	65.5

Table 3 Percentages of neuroendocrine carcinoma (NEC) among pancreatic and gastrointestinal neuroendocrine tumors in 2010

	Number of patients	Percentage (%)
(a) Total PNETs	95/1,273	7.5
Functioning PNETs	14/439	3.2
Insulinoma	5/266	1.9
Gastrinoma	6/104	5.8
Glucagonoma	1/42	2.4
VIPoma	0/8	0
Somatostatinoma	0/4	0
Others	2/17	11.8
Non-functioning PNETs	81/834	9.7
(b) Total GI-NETs	130/2,093	6.2
Foregut	93/737	12.6
Midgut	7/77	9.1
Hindgut	30/1,279	2.3

PNETs pancreatic neuroendocrine tumors, *GI-NETs* gastrointestinal neuroendocrine tumors

frequent functioning PNETs were insulinoma (20.9 %) followed by gastrinoma (8.2 %). Glucagonoma, VIPoma, and somatostatinoma had low frequencies of 3.2, 0.6, and 0.3 %, respectively.

Histopathological distribution of PNETs in Japan in 2010 (Table 3a)

The histological survey was conducted according to the 2010 WHO classification. This survey comprised 2 parts: one was for NETs (G1/G2) and the other for neuroendocrine carcinoma (NEC; small-cell or large-cell type). The frequency of NECs among all PNETs was 7.5 %. The frequency of NECs among NF-PNETs was high at the rate of 9.7 % compared with that among functioning PNETs at the rate of 3.2 %.

Percentages of distant metastases and association of MEN-1 in PNETs (Table 4)

Among the patients with PNETs, 19.9 % exhibited distant metastases at initial diagnosis; the percentages among functioning PNETs and NF-PNETs were 16.9 % and 21.3 %, respectively. Among functioning PNETs, gastrinoma accounted for 30.2 %, whereas insulinoma accounted for 9.3 %. With regard to the grade of WHO calcification, the percentage of distant metastases in patients with NEC at initial diagnosis was high at the rate of 46.3 % compared with that in patients with NET G1/G2 at the rate of 12.9 %. Especially, NF-PNETs patients with NEC was the most prevalent at the rate of 51.9 %.

On the other hand, complications with MEN-1 accounted for 4.3 % of all PNETs (4.9 % of functioning PNETs and 4.0 % of NF-PNETs). The percentage of complications with MEN-1 among cases of gastrinoma was high (16.3 %) but low among cases of insulinoma (0.8 %).

Epidemiology of GI-NETs in Japan in 2010

Epidemiology (Table 5)

The present survey estimated a total of 8,088 people (95 % CI 5,669–10,507) were treated for GI-NETs in 2010. The

total numbers of patients treated for foregut, midgut, and hindgut tumors in this group were 2,107 (95 % CI 1,189–3,028), 290 (95 % CI 271–349), and 5,690 (95 % CI 3,583–7,797), respectively. There were approximately 1.8 times as many patients in 2010 as those in 2005. The overall prevalence of GI-NETs was 6.42 per 100,000 people (95 % CI 4.50–8.34). The overall prevalences of foregut, midgut, and hindgut tumors were 1.67 (95 % CI 0.94–2.40), 0.23 (95 % CI 0.18–0.28), and 4.52 per 100,000 people (95 % CI 3.17–5.87), respectively. The locations of GI-NETs varied: 26.1, 3.6, and 70.3 % were in the foregut, midgut, and hindgut, respectively. Similar to the survey results from 2005, the frequency of midgut NETs was very low in Japan relative to that in Western nations. Meanwhile, the incidence rate of GI-NETs in 2010 was estimated to be 3.51 per 100,000 people (95 % CI 2.50–4.53); the incidence rates of foregut, midgut, and hindgut tumors in this group were 1.20 (95 % CI 0.48–1.91), 0.15 (95 % CI 0.12–0.18), and 2.12 per 100,000 people (95 % CI 1.56–2.67), respectively. Although the incidence rates of foregut and hindgut tumors clearly increased since 2005, no change in the incidence rate of midgut tumors was observed.

Table 4 Percentages of distant metastases and associated MEN-1 in pancreatic neuroendocrine tumors (PNETs) in 2010

	Distant metastases (%)			Associated MEN-1 (%)
	Total	NET G1/G2	NEC	
Total PNETs	19.9	12.9	46.3	4.3
Functioning PNETs	16.9	17.2	14.3	4.9
Insulinoma	9.3	9.7	0	0.8
Gastrinoma	30.2	32.4	10.7	16.3
Glucagonoma	8.3	9.1	0	8.3
VIPoma	80.0	80.0	0	0
Somatostatinoma	100	100	0	0
Others	25.0	0	50	0
Non-functioning PNETs	21.3	12.9	51.9	4.0

MEN-1 multiple endocrine neoplasia type 1

Histopathological distribution of GI-NETs in Japan in 2010 (Table 3b)

The frequency of NEC among all GI-NETs was 6.2 %. NEC was most common among foregut NETs (12.6 %) followed by midgut NETs (9.1 %) and hindgut NETs (2.3 %).

Percentages of distant metastases and association between MEN-1 and frequency of carcinoid syndrome in GI-NETs (Table 6)

Among all patients with GI-NETs, distant metastases were observed at initial diagnosis in 6.0 %. Regarding location, midgut NETs were the most common (9.8 %) followed by foregut NETs (8.6 %) and hindgut NETs (3.5 %). With regard with the grade of WHO calcification, the percentage of distant metastases in patients with NEC at initial diagnosis was high at the rate of 32.3 % compared with that in patients with NET G1/G2 at the rate of 2.7 %. Especially, foregut NETs patients with NEC was the most prevalent at the rate of 40.9 %.

Meanwhile, complications with MEN-1 were observed in 0.7 % of all GI-NETs. Regarding location, they were observed in 0.7, 0, and 0.2 % of foregut, midgut, and

Table 5 The trends of epidemiology of gastrointestinal neuroendocrine tumors (GI-NETs) from 2005 to 2010 in Japan

	2005*	2010
Total number of patients treated for GI-NETs	4,406 (95 % CI 3,321–5,420)	8,088 (95 % CI 5,669–10,507)
Foregut	1,338 (95 % CI 1,009–1,640)	2,107 (95 % CI 1,189–3,028)
Midgut	423 (95 % CI 319–520)	290 (95 % CI 271–349)
Hindgut	2,645 (95 % CI 1,994–3,254)	5,690 (95 % CI 3,583–7,797)
Overall prevalence of GI-NETs (per 100,000 population)	3.45 (95 % CI 1.93–4.24)	6.42 (95 % CI 4.50–8.34)
Foregut	1.05 (95 % CI 0.59–1.28)	1.67 (95 % CI 0.94–2.40)
Midgut	0.33 (95 % CI 0.18–0.41)	0.23 (95 % CI 0.18–0.28)
Hindgut	2.07 (95 % CI 1.56–2.55)	4.52 (95 % CI 3.17–5.87)
Incidence rate of GI-NETs (per 100,000 population)	2.10 (95 % CI 1.56–2.54)	3.51 (95 % CI 2.50–4.53)
Foregut	0.64 (95 % CI 0.48–0.77)	1.20 (95 % CI 0.48–1.91)
Midgut	0.20 (95 % CI 0.15–0.24)	0.15 (95 % CI 0.12–0.18)
Hindgut	1.26 (95 % CI 0.94–1.52)	2.12 (95 % CI 1.56–2.67)

*Data modified from reference [5]

95 % CI 95 % confidence interval; *Foregut* esophagus, stomach and duodenum; *Midgut* jejunum, ileum and vermiform appendix; *Hindgut* large intestine and colon

Table 6 Percentages of distant metastases, associated MEN-1 and carcinoid syndrome in gastrointestinal neuroendocrine tumors (GI-NETs) in 2010

	Distant metastases (%)			Associated MEN-1 (%)	Carcinoid syndrome (%)
	Total	NET G1/G2	NEC		
Total GI-NETs	6.0	2.7	32.3	0.42	3.2
Foregut	8.6	1.8	40.9	0.72	1.1
Midgut	9.8	5.9	28.6	0	17.1
Hindgut	3.5	2.2	26.7	0.16	4.2

MEN-1 multiple endocrine neoplasia type 1

hindgut NETs, respectively; this indicates complications with MEN-1 in GI-NETs are rare in Japan.

In addition, the frequency of carcinoid syndrome in patients with GI-NETs was 3.2 %. Thus, carcinoid syndrome in GI-NETs is observed less frequently in Japan than Western nations. Regarding location, midgut NETs were the most common (17.1 %) followed by foregut NETs (4.2 %) and hindgut NETs (1.1 %).

Discussion

The second nationwide epidemiological survey of patients with GEP-NETs was conducted in Japan in 2010, and the data were compared with those from 2005 to elucidate epidemiological changes.

An estimated 3,379 patients received treatment for PNETs from January 1 to December 31, 2010 in Japan; therefore, the prevalence of PNETs is about 2.69 per 100,000 people. In 2005, these figures were 2,845 and 2.23 per 100,000 people, respectively, indicating an approximately 1.2-fold increase in the number of patients. The incidences of new-onset PNETs in 2005 and 2010 were about 1.01 and 1.27 per 100,000 people, respectively, indicating a 5-year increase in the incidence of new-onset PNETs. Interestingly, the percentage of NF-PNETs increased from 42.8 % in 2005 to 65.5 % in 2010, approaching that of Western nations [2, 7, 8]. There are 2 possible reasons for this. First, the disease concept of NETs disseminated among general clinicians; that is, clinicians have become accustomed to keeping PNETs in mind when treating pancreatic tumors. Second, the availability of endoscopic ultrasonography (EUS), which is useful for the diagnosis of pancreatic diseases [9, 10], has made endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) easy to perform for pancreatic tumors, which were merely being followed-up before; thus, the pathological diagnosis of PNETs has become more accurate [11, 12].

An estimated 8,088 people received treatment for GI-NETs in Japan in 2010, which means the prevalence of patients with this disease was about 6.42 per 100,000 people; in 2005, these figures were 4,406 and 3.45 per 100,000 people, respectively, indicating a 1.8-fold increase in the number of patients with this disease. In addition, the incidence rate of new-onset GI-NETs increased from about 2.1 per 100,000 people in 2005 to about 3.51 per 100,000 people in 2010.

Similar to the 2005 survey, few patients had midgut NETs and the locations of GI-NETs varied: 26.1, 3.6, and 70.3 % in the foregut, midgut, and hindgut, respectively. In Western nations, 30–60 % of GEP-NETs are derived from midgut [2, 13, 14] in contrast to the Japanese data. The epidemiology of GEP-NETs was recently reported in Asian nations including Taiwan [15], China [16], and Korea [17, 18]. Interestingly, the prevalence of patients with midgut NETs in these nations is low like Japan, indicating ethnic differences between Asians and Western populations.

The present study involved a survey conducted according to the 2010 WHO classification [6]. The 2010 WHO classification distinguishes between well-differentiated NETs and poorly differentiated NECs of small- or large-cell type. NETs are further divided with respect to Ki-67 index: NET G1 and NET G2. Before the present survey was conducted, the frequency of NEC among GEP-NETs in Japan was not clear. A Korean study [17] reports that the frequency of NECs among all GEP-NETs is 2.84 %. Meanwhile, in the present survey, the frequency of NEC among all GEP-NETs in Japan was 6.7 % (225/3,366). Interestingly, the frequency of NEC in NF-PNETs was 9.7 %, which is substantially higher than that reported in Western nations, where NEC in NF-PNETs is uncommon [8]. However, with regard to the grade of WHO calcification, the percentage of distant metastases in patients with NEC at initial diagnosis was high compared with that in patients with NET G1/G2. Especially, NF-PNETs patients with NEC was the most prevalent at the rate of 51.9 %. On the other hand, the frequency of NEC among all GI-NETs was 6.2 % in the present study; the common types were foregut NEC (12.6 %), midgut NETs (9.1 %), and hindgut NETs (2.3 %). Similarilly, the percentage of distant metastases in patients with NEC at initial diagnosis was high compared with that in patients with NET G1/G2.

According to the US SEER study, distant metastases are present in 64 % of PNETs followed by cecal, colonic, and small-intestinal NETs in 44, 32, and 30 % of PNETs, respectively [6]. In European and American referral centers, up to 77 and 91 % of patients with PNETs and intestinal NETs [19–22] present with distant metastases at initial diagnosis, respectively [13]. In the present Japanese study, patients in whom distant metastases were observed at initial diagnosis accounted for 19.9 % of PNETs and

6.0 % of GI-NETs. Regarding the location of GI-NETs, midgut NETs were the most common (9.8 %) followed by foregut NETs (9.8 %) and hindgut NETs (3.5 %); however, these frequencies are substantially lower than those reported in Western nations. Furthermore, as shown in Table 6, the frequency of carcinoid syndrome in patients with GI-NETs is low (3.2 %) compared to that reported in Western nations, suggesting ethnic differences.

At present, 4 genetic diseases—MEN-1, von Hippel-Lindau (VHL) disease, von Recklinghausen disease, and tuberous sclerosis—are thought to be associated with NETs [23]. As for PNETs complicated with MEN-1 [24, 25] or VHL, [26], screening must be performed at the initial diagnosis of PNETs because of different surveillance methods and treatment guidelines. MEN-1 is reported to be complicated with NF-PNETs, gastrinoma, and insulinoma at frequencies of about 80 %, 50 %, and 20 %, respectively [23]. On the other hand, 20–25 % of gastrinomas and 4–5 % of insulinomas are reported to be complicated with MEN-1 [27]. The rate of MEN-1 association in functional PNETS in the present study (4.9 %) does not differ from that reported in Western nations [27, 28]. However, MEN-1 associated with NF-PNETs was observed in only 4.0 % of cases in Japan. Furthermore, the presence of MEN-1 in GI-NETs in the present study was only 0.7 %, whereas approximately 30 % of NF-PETs are reported to be associated with MEN-1 in Western nations [28]. The difference in the frequencies of MEN-1 in NF-PETs and GI-NETs between Japan and Western nations may be due to ethnic differences as well.

There is currently no consensus regarding antitumor chemotherapy drugs against advanced GEP-NETs in Japan, and most treatment regimens are not covered by insurance. Global clinical studies on various molecularly targeted drugs against GEP-NETs were recently conducted. The results show everolimus [29, 30], an mTOR inhibitor, and sunitinib [31, 32], a multikinase inhibitor, are effective against advanced PNETs (NET G1/G2); in addition, octreotide LAR was shown to be effective against midgut-derived, metastatic, well-differentiated NETs in 2009 (PROMID study) [33]. These drugs have become reimbursable as antitumor drugs for treating advanced GI-NETs in Japan. Regarding NET, functionality, invasion depth, and the presence or absence of metastases must be correctly evaluated and treatment administered on the basis of the degrees of differentiation and malignancy of the tumor [4, 34–36]. Although surgical total excision is the standard treatment [37], some studies report that when radical treatment is difficult, debulking surgery of primary lesions and liver metastatic lesions effectively alleviate symptoms and improve prognosis [4, 34, 37]. On the other hand, in cases of unresectable advanced tumors, treatment aiming to improve prognosis by inhibiting tumor growth and improving clinical symptoms is necessary [8, 13, 27]. For this purpose, it is important to understand patient backgrounds, particularly epidemiological background, and be aware of the epidemiological differences between Japanese and Western populations. Thus, the results of the present epidemiological survey investigating the 5-year changes in GEP-NETs in Japan will be invaluable to clinicians.

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Serum chromogranin A is a useful marker for Japanese patients with pancreatic neuroendocrine tumors

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Although chromogranin A (CGA) is a useful marker for pancreatic neuroendocrine tumors (pNET) in the West, its usefulness in Japanese populations is unclear. To assess this, we evaluated the serum CGA levels in 189 patients with various pancreatic diseases, including proven pNET (n = 69), pancreatic cancer (PC) (n = 50), chronic pancreatitis (CP) (n = 50) and autoimmune pancreatitis (AIP) (n = 20), and 112 normal controls (controls) using an ELISA kit. The mean CGA level of patients with pNET was significantly higher than any of the other groups (407.8 ± 984.6 ng/mL [pNET] *vs* 91.8 ± 101.8 ng/mL [PC], 93.6 ± 57.5 ng/mL [CP], 69.9 ± 52.4 ng/mL [AIP] and 62.5 ± 48.3 ng/mL [controls]). Limiting the analysis to patients not using proton pump inhibitors (PPI), the CGA level of patients with PC or CP was not significantly different compared with the controls. Discriminant analysis revealed that the best cut-off value of CGA to distinguish patients with pNET from the controls was 78.7 ng/mL, with a sensitivity and specificity of 53.6% and 78.6%, respectively. In patients with pNET, significant factors associating with elevated CGA levels were tumor classification, tumor size, and the presence of liver metastases in univariate analysis as well as PPI use and the presence of liver metastases in multivariate analysis. We show that CGA is a useful marker for diagnosing pNET in Japanese populations and for distinguishing patients with pNET from patients with other pancreatic diseases. The increased use of CGA in Japan will likely be a helpful tool in managing these patients, as found in the West.

Pancreatic neuroendocrine tumors (pNET) are uncommon tumors that are derived from the diffuse neuroendocrine cell system.[1] It is typically an indolent slow-growing tumor.[2]

However, pNET are receiving increasing attention worldwide and are increasingly being seen in clinical practice. This is because the prevalence of pNET has been increasing over the past three decades in a number of Western countries, which could be due to the increased use of endoscopic or imaging procedures, increased clinical awareness or a real increase in the incidence.[2,3] Recent data suggest that a similar trend is also true in Japan. A nationwide epidemiological study in 2005 revealed the current status of pNET in Japan.[4] A second nationwide epidemiological study in 2010 revealed that the number of patients with pNET is increasing in Japan.[5] These reports also reveal some differences in the epidemiology between Japan and Western countries.[4,5] Unfortunately, the survival of patients with pNET, similarly to those with other gastrointestinal neuroendocrine tumors (GI-NET), has not increased,[2] which is likely because patients continue to be diagnosed late in their disease course, with an average delay in diagnosis of 5–8 years.[2,6]

Pancreatic neuroendocrine tumors are receiving increased attention not only because their frequency is increasing but also because it is increasingly recognized that a significant proportion are malignant and require treatment.[7] Although pNET have a less aggressive course than adenocarcinomas, these malignant forms are associated with considerable morbidity. Furthermore, because they have a different pathogenesis to typical adenocarcinomas, they require a different therapeutic approach as well as a different approach in their diagnosis. During the past several years, new therapeutic agents for patients with pNET have been developed that can affect malignant progression, and it is important that patients with pNET are recognized. Both everolimus and sunitinib have demonstrated the ability to prolong the progression-free survival in patients with pNET.[7–9] These agents provide a similar benefit in Japanese patients with pNET and are now approved for use in Japan.[10,11] These reports also show differences in the response to drugs between patients with pNET in Japan and those in Western countries.[10,11]

For the reasons outlined above, it is increasingly important in Japan, as in Western countries, to have reliable methods that are generally available for both diagnosing and assessing the results of treatments in patients with pNET as well as with other GI-NET, such as carcinoids. Currently, imaging modalities are generally used, but they are sometimes unfavorable for

patients, frequently involve exposure to radiation and can be expensive. For some cancers, tumor markers have proven useful for this purpose (e.g. calcitonin for medullary thyroid tumor and prostate-specific antigen [PSA] for prostate cancer),[12,13] and, in the case of pNET as well as GI-NET, from studies in the West, assessment of chromogranin A (CGA) in the serum/plasma shows the most promise.[14,15]

In the USA and other Western countries, CGA is broadly used as a marker for both diagnosing and monitoring the response to therapy of patients with pNET as well as with GI-NET.[1,14–17] CGA is a hydrophilic, acidic protein that consists of 439 amino acids.[14,15,17–19] CGA is present in chromaffin granules of neuroendocrine cells in both normal tissues and in neuroendocrine tumors (NET).[17–19] Therefore, CGA is used as a general marker for all NET.

We should take into consideration that several clinical conditions, other than NET, influence the elevation of the serum CGA level when we assess the serum CGA levels in patients with pNET. For example, the elevation (especially low level increases) of serum CGA levels can occur in several non-neoplastic conditions (e.g. inflammatory bowel syndrome[20] and chronic renal failure[21,22]) or certain adenocarcinomas (e.g. breast cancer[23] and hepatocellular carcinoma[22]).[14] Particularly important for the diagnosis and assessment of pNET are reports in other countries that the serum CGA levels can be elevated in patients with other pancreatic diseases, such as pancreatic cancer (PC) or chronic pancreatitis (CP).[24] In some cases, we have difficulty distinguishing pNET from these diseases, which affects the provision of the correct therapy for patients with pNET. It is important to know the difference in the serum CGA level between patients with pNET and these diseases.

As described above, there are differences between Japan and Western countries with respect to the epidemiology and therapeutic effects in patients with pNET. For this reason, there might also be differences in the serum CGA level by race. In fact, some tumor markers have been reported to have differences by race.[13,25] However, the serum CGA assessment has not previously been shown to be a useful marker for pNET in Japan. In addition, the serum CGA level in patients with other pancreatic diseases and whether CGA is useful for distinguishing pNET from these diseases in Japan have not previously been studied. Furthermore, the standard levels of CGA for Japanese people have not been well-studied. To address these issues, in the present study we studied both a group of Japanese normal controls, as well as patients with pNET and various pancreatic diseases. Our studies demonstrate that the serum CGA level is a useful marker for Japanese patients in diagnosing pNET and could be used in Japan to distinguish these patients from patients with other pancreatic diseases.

Material and Methods

We evaluated serum samples of 189 patients with pNET ($n = 69$), PC ($n = 50$), CP ($n = 50$) and autoimmune pancreatitis (AIP) ($n = 20$) who visited our institution from April 2008 to September 2012. All patients with pNET were histologically diagnosed with well-differentiated tumors corresponding to NET grade G1 or G2 according to the World Health Organization 2010 classification.[26] In 89.9% of patients, Ki67 value determination was performed and found to be G1 or G2. In the remaining 10.1% of patients, cytology was performed, but Ki67 value was not determined, and they were established to be well-differentiated tumors corresponding to G1 or G2. Patients with neuroendocrine carcinoma were excluded from this study. Each functional pNET was diagnosed by the existence of symptoms arising from oversecretion of each hormone. All patients with PC were histologically verified. All patients with CP or AIP were diagnosed using their standard diagnostic criteria in Japan, respectively.[27,28] We also evaluated serum samples of 112 controls. All controls confirmed that they were not using proton pump inhibitors (PPI), which can elevate serum CGA levels due to the gastric enterochromaffin-like cell changes these agents can cause,[15,17] and that they were not suffering from diseases of other organs, including the pancreas.

The study protocol was approved by the ethics committee at Kyushu University and written informed consent was obtained from all patients.

Blood samples were collected from each patient while fasting, centrifuged to obtain serum samples and stored at −80°C until assay. The serum CGA level was measured by using Chromoa (CIS Bioassays, GIF-SUR-YVETTE, France), which is an ELISA kit. We confirmed that the intra-assay and inter-assay coefficients of variation are 5% and 7%, respectively.

Differences in patient characteristics between each group were evaluated by 2×2 χ^2-square test, Student *t*-test or Fisher's exact test. Differences in the serum CGA level between each group were evaluated using Scheffe's multiple comparisons test. Correlation coefficients were calculated to evaluate the correlations between the serum CGA and patient characteristics or other tumor markers. To determine a best cut-off value of the serum CGA to distinguish patients with pNET from the controls, discriminant function was calculated and a receiver operating characteristic curve was constructed. Univariate or multivariate analysis was conducted to determine the association between patient characteristics and elevated serum CGA level. $P < 0.05$ was considered statistically significant.

Results

Patient characteristics. The patient characteristics of each group are shown in Table 1. There were no significant differences between each group in terms of age and gender. In the pNET group, tumors consisted of non-functioning tumors (56.5%) with 43.5% of the functional tumor consisting primarily of gastrinomas (24.6%) and insulinomas (14.5%). All tumors were well-differentiated with histological grades of NET-G1 (58.0%) and NET-G2 (31.9%); in the remainder (10.1%), the histology was verified as well-differentiated (G1/G2) by cytology without an exact Ki67 value. In 48 patients (69.5%), a primary tumor remained in the pancreas at the time of the CGA measurement, whereas in 21 patients (30.5%), a primary tumor was resected from the pancreas and metastatic lesions were present at the time of CGA measurement. Among the patients with a primary tumor remaining in the pancreas, the maximum diameter of the primary tumor was <2 cm in 33 patients (47.8%) and >2 cm in 15 patients (21.7%).

The proportions of the presence of liver metastases and multiple endocrine neoplasia type 1 (MEN-1) in the pNET group were 40.6% and 8.7%, respectively.

Serum chromogranin A level. The measurement result of the serum CGA level in each group is shown in Table 2. The mean serum CGA level of patients with pNET was 6.5-fold higher than in the controls and was significantly higher compared with the controls ($P < 0.01$). This level was also 4.5-fold higher than those of other groups and was significantly higher compared with those in the PC ($P < 0.05$) and CP ($P < 0.05$)

Table 1. Patient characteristics of this study

Characteristics	pNET	PC	CP	AIP	Normal	*P*-value
Number	69	50	50	20	112	
Sex (%)						
Male	39 (56.5)	28 (56.0)	30 (60.0)	17 (85.0)	67 (59.8)	0.709
Female	30 (43.5)	22 (44.0)	20 (40.0)	3 (15.0)	45 (40.2)	
Age (years)						
Mean ± SD	57.5 ± 13.9	63.8 ± 9.5	53.0 ± 14.0	63.6 ± 11.4	56.5 ± 14.3	0.286
Range	(20–85)	(46–84)	(25–75)	(35–75)	(26–99)	
PPI use (%)						
Yes	19 (27.5)	19 (38.0)	28 (56.0)	9 (45.0)	0 (0)	<0.0001*
No	50 (72.5)	31 (62.0)	22 (44.0)	11 (55.0)	112 (100)	
Tumor classification (%)						
Non-functioning	39 (56.5)					
Functioning	30 (43.5)					
Gastrinoma	17 (24.6)					
Insulinoma	10 (14.5)					
Others†	3 (4.3)					
Histological grade						
G1	40 (58.0)					
G2	22 (31.9)					
G1 or G2‡ (an exact Ki67 not determined)	7 (10.1)					
Tumor size (pancreas) (%)						
<2 cm	33 (47.8)					
>2 cm	15 (21.7)					
Postoperative	21 (30.5)					
Liver metastasis (%)						
Yes	28 (40.6)					
No	41 (59.4)					
Presence of MEN-1 (%)						
Yes	6 (8.7)					
No	63 (91.3)					

P-value was calculated using 2 × 2 χ^2-test or Student *t*-test or Fisher's exact test. *Significant difference using Fisher's exact test. †Others comprise of a glucagonoma, a somatostatinoma and a VIPoma. ‡Cytology was performed but not determined Ki67 value and diagnosed with well-differentiated tumor which corresponds to NET G1 or G2 according to the WHO 2010 classification. AIP, autoimmune pancreatitis; CP, chronic pancreatitis; MEN-1, multiple endocrine neoplasia type 1; PC, pancreatic cancer; pNET, pancreatic neuroendocrine tumor; PPI, proton pump inhibitor.

groups but not in the AIP group ($P = 0.10$), which is most likely because of the small sample size. The mean serum CGA level of all patients with PC or CP was 1.5-fold higher than in the controls, but the effect was not significant ($P = 0.99$, respectively). Next, we conducted a subgroup analysis based on PPI use because PPI can elevate the serum CGA level, and patients with pancreatic diseases often take PPI. The serum CGA level of patients using PPI was significantly higher than that of patients not using PPI in the in the PC ($P < 0.05$), CP ($P < 0.05$) and AIP ($P < 0.05$) groups but not in the pNET group ($P = 0.21$). In patients not using PPI, the mean serum CGA level of patients with pNET was 7.1-fold higher than in the controls, which was significantly different from the controls ($P < 0.01$) and patients in the PC group ($P < 0.05$) but not patients in the CP ($P = 0.11$) and AIP ($P = 0.28$) groups. Furthermore, the serum CGA levels of patients in the PC, CP and AIP groups not taking PPI were not different from the controls ($P = 0.93$, $P = 0.90$ and $P = 0.93$, respectively). The distribution of the serum CGA levels in each group is shown in Figure 1.

Regression analyses in pancreatic neuroendocrine tumor group. A more in-depth analysis was performed on patients with pNET. First, we performed regression analysis to clarify the factors associated with an elevation of the serum CGA level. This analysis revealed that the presence of liver metastases was the only associated factor for either single or multiple regression analyses (Table 3). Gender almost reached significance in the single regression analysis ($P = 0.063$) and the multiple regression analysis ($P = 0.061$), showing a trend for females to have higher values, but it did not reach significance with this limited number of patients (Table 3).

Discriminant analysis in pancreatic neuroendocrine tumor group from normal. Next, a discriminant function was calculated to set the best cut-off value of CGA to distinguish patients with pNET from controls. The results showed that the best cut-off value of CGA for distinguishing between patients with pNET and controls was 78.7 ng/mL, with a sensitivity and specificity of 53.6% and 78.6%, respectively (Fig. 2a). A receiver operating characteristic curve was constructed to confirm the results (Fig. 2b).

Univariate and multivariate analyses of pancreatic neuroendocrine tumor group. Using the cut-off value calculated above, patients with pNET could be divided into two subgroups: one group with a serum CGA level above the cut-off value and the other with a serum CGA level below the cut-off value. Univariate analysis revealed that the tumor classification, the tumor size and the presence of liver metastases were significantly associated with the serum CGA levels above this cut-off value