Expression of Somatostatin Receptor Type 2A and PTEN in Neuroendocrine Neoplasms is Associated with Tumor Grade but not with Site of Origin

Hideo Wada · Katsuya Matsuda · Yuko Akazawa · Yuka Yamaguchi · Shiro Miura · Nozomi Ueki · Akira Kinoshita · Koh-ichiro Yoshiura · Hisayoshi Kondo · Masahiro Ito · Takeshi Nagayasu · Masahiro Nakashima

Hideo Wada · Takeshi Nagayasu

Division of Surgical Oncology, Nagasaki University Graduate School of Biomedical Sciences

1-7-1 Sakamoto, Nagasaki 852 8501, Japan

Hideo Wada · Katsuya Matsuda · Nozomi Ueki · Masahiro Nakashima

Department of Tumor and Diagnostic Pathology, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852 8523, Japan

Yuko Akazawa

Department of Gastroenterology and Hepatology, Nagasaki University Hospital

1-7-1 Sakamoto, Nagasaki 852 8501, Japan

Yuka Yamaguchi

Medical Student Research Programme, Nagasaki University School of Medicine

1-12-4 Sakamoto, Nagasaki 852 8523, Japan

Shiro Miura · Masahiro Nakashima

Tissue and Histopathology Section, Atomic Bomb Disease Institute, Nagasaki University

1-12-4 Sakamoto, Nagasaki 852 8523, Japan

Akira Kinoshita · Koh-ichiro Yoshiura

Department of Human Genetics, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852 8523, Japan

Hisayoshi Kondo

Biostatic section, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences

1-12-4 Sakamoto, Nagasaki 852 8523, Japan

Masahiro Ito

Department of Pathology, National Hospital Organization Nagasaki Medical Center

2-1001-1 Kubara, Nagasaki 856 8562, Japan

Address for correspondence: M Nakashima, Department of Tumor and Diagnostic Pathology, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan.

Phone: +81-95-819-7107; Fax: +81-95-819-7108; e-mail: moemoe@nagasaki-u.ac.jp

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Abstract Neuroendocrine neoplasms (NENs) are derived from endocrine cells in various organs and share common morphological features. This study aimed to clarify whether NENs of different organs are comparable at the molecular pathologic level. We retrospectively collected 99 cases of NENs from gastro-entero-pancreatic, lung, and other organs and reclassified these according to identical criteria. Grade, site, and molecular expression profile including NE markers, Ki-67, p53, somatostatin receptor type 2A (SSTR2A), and phosphatase and tensin homolog (PTEN) were compared. PTEN immunoreactivity was also compared with genomic copy number by fluorescence *in situ* hybridization (FISH) and droplet digital polymerase chain reaction (ddPCR). No significant differences were observed in the immunoreactivities of NE markers, p53, SSTR2A, or PTEN expression in NENs between the different organ sites. PTEN and p53 functional inactivation along with the loss of membranous SSTR2A expression appeared to be commonly involved in high grade NEN. FISH results were significantly correlated with the level of PTEN immunoreactivity and with the findings of ddPCR analyses. The demonstration that these tumors are comparable at the molecular level will likely contribute to the broadening of therapeutic options such as the use of somatostatin analogues and mTOR inhibitors against NENs regardless of the affected organ, whereas molecular characterization of tumor grade will be useful for determining treatment strategy.

Keywords: Neuroendocrine neoplasm · PTEN · SSTR2A · p53 · FISH · ddPCR

Introduction

Neuroendocrine neoplasms (NENs) occur in various organs including the gastrointestinal tract, pancreas, and the lung, and exhibit a higher incidence than previously realized [1]. NENs are derived from endocrine cells and share common morphological features such as growth patterns (e.g., palisading, trabecular, and rosette-like arrangements) that suggest neuroendocrine differentiation; uniform cytological features with eosinophilic, finely granular cytoplasm; and nuclei with the finely granular chromatin pattern of tumor cells [2, 3]. The immunohistochemical findings of chromogranin A (CgA), synaptophysin (Syn), CD56, and neuron-specific enolase (NSE) in these tumors also support their endocrine origin. Surgical resection is the only common radical therapy available whereas for inoperable metastasized cases, chemotherapy and biotherapy represent the primary treatment modalities.

Loss of heterozygosity (LOH) of the tumor suppressor phosphatase and tensin homolog (PTEN) concurrent to intrachromosomal 10 deletion is a common genetic event in small cell neuroendocrine carcinomas (NECs) of the lung and gastrointestinal tract [4. 5], and we previously described a high-grade neuroendocrine tumor (NET) of the thyroid carrying PTEN alterations [6]. For such tumors, the tyrosine kinase inhibitor sunitinib and the mTOR inhibitor everolimus have shown antitumor effects and improved prognosis [7–9]. Notably, low PTEN expression is also linked with clinical outcomes in patients with pancreatic NEN [10] and with sensitivity to mTOR inhibitors in other solid cancers [11,12]. However, the use of mTOR inhibitors has been limited only to advanced pancreatic NENs. In addition, somatostatin receptor subtype 2A (SSTR2A), which is known as an inhibitor of proliferation, is frequently overexpressed in NENs in multiple endocrine neoplasias [13]. Consequently, somatostatin analogues have shown antitumor effects, prolonging the time to disease progression in patients with midgut NENs, especially in well-differentiated NEN and SSTR2A-positive patients [14]. However, their use has been limited to midgut NENs and is not

currently applied to NENs originating in other organs.

Despite the morphologic similarities of NENs, distinct diagnostic criteria and treatment options vary across organs. Among gastro-entero-pancreatic (GEP) NENs, well- and poorly differentiated NETs are assigned to NET G1/G2 and NEC G3, respectively, according to the 2010 WHO classification based on morphological criteria; the assessment of the proliferative fraction is based on the mitotic count [3]. In the lungs, however, the major categories of morphologically identifiable NENs are generally defined as typical carcinoid, atypical carcinoid, large cell neuroendocrine carcinoma (LCNEC), and small cell carcinoma (SCC), according to the mitotic activity and the presence or absence of necrosis [15]. Thus, although different diagnostic criteria and treatments are applied according to sites of NEN origin, we hypothesize that marked molecular pathological similarities exist among these tumors such as SSTR2A and PTEN expression profiles, which can affect disease outcome.

This study aimed to clarify whether NENs of different organs are comparable at the molecular pathologic level when identical diagnostic criteria, such as the 2010 WHO GEP-NEN classification grading system, are used. In particular, we are interested in the comparative profiles of SSTR2A and PTEN expression, which can serve as predictors of sensitivity for somatostatin analogue and mTOR inhibitor treatment, respectively. By demonstrating whether these tumors are comparable at the molecular pathologic level, the current study will inform whether the biotherapeutic options for NEN treatment would likely be efficacious for and thus could be broadened to include tumors regardless of the organ of origin.

Materials and Methods

Tissue Samples

A total of 99 archival NEN tissue samples, originating in divergent organs including 49 GEP, 37 lung, and 13 other,

were available. All samples were formalin-fixed paraffin-embedded (FFPE) tissues that were surgically or endoscopically resected between 1991 through 2014. The pathological diagnoses of all cases were independently reviewed by three pathologists (H.W., M.I., and M.N.) and reclassified into three categories, NET G1, G2, and G3 according to the grading system of the GEP-NEN 2010 WHO classification [3]. Representative images of these cases are depicted in Fig. 1. The clinicopathologic profiles of the tissue samples in this study are summarized in Table 1. To further validate whether the GEP-NET 2010 WHO classification is applicable to multi-organ NENs regardless of tumor-site, we evaluated overall survival (OS) rate at 36 months after tumor resection. The present study was an un-linkable-anonymized study strictly following the principles established in the Declaration of Helsinki and was approved by the Committee for Ethical Issues of Nagasaki University Graduate School of Biomedical Sciences (Date of approval; Aug. 20, 2015, Protocol No. 15682035). As this was a retrospective research study involving minimal risk to the participants, detailed information of the research was released to the public on the institution's homepage (http://www-sdc.med.nagasaki-u.ac.jp/pathology/index.html) following the guidelines of the Ethical Committee's official disclosure system.

Immunohistochemistry

After antigen retrieval by heating in a microwave, the sections were immersed in 0.3% H_2O_2 solution to block endogenous peroxidase activity. Tissue sections were then reacted with primary antibodies against neuroendocrine markers including anti-CgA (monoclonal, Novocastra, Newcastle upon Tyne, UK), anti-Syn (monoclonal, Nichirei Biosciences Inc., Tokyo, Japan), anti-CD56 (monoclonal, Nichirei Biosciences Inc.), and anti-NSE (monoclonal, DakoCytomation, Glostrup, Denmark). In addition, immunohistochemical staining was performed for Ki-67 (MIB-1,

monoclonal, DakoCytomation), p53 (monoclonal, DakoCytomation), PTEN (monoclonal, DakoCytomation), and SSTR2A (polyclonal, Gramsch Laboratories, Schwabhausen, Germany). Histofine Simple Stain^{TM} MAX PO (MULTI) (Nichirei Biosciences Inc.) was used for detection according to manufacturer instruction.

Evaluation of Immunofluorescence Results

CgA, Syn, and NSE, CD 56, or p53 expression was classified as positive if clear cytoplasmic staining, clear membranous staining, or clear nuclear staining was observed in > 30% of tumor cells, respectively. The Ki-67 labeling index (LI) was calculated by according to the 2010 WHO GEP-NEN grading system [3]. For SSTR2A expression, a semi-quantitative scoring system was applied as described previously [16], considering both subcellular localization and extent of staining as follows: 0, absence of immunoreactivity; 1, pure cytoplasmic immunoreactivity, either focal or diffuse; 2, membranous reactivity in <50% of tumor cells, irrespective of cytoplasmic staining; or 3, circumferential membranous reactivity in >50% of tumor cells, irrespective of cytoplasmic staining [16]. Immunohistochemical PTEN expression was classified according to the staining range of cells as follows: negative, no staining; focal, partial staining; diffuse, cytoplasmic staining in all tumor cells.

Dual-Color Interphase Fluorescence *In Situ* Hybridization (FISH) for *PTEN*

A dual-color FISH assay was performed on the sections to confirm *PTEN* gene expression. The LSI *PTEN* SpectrumOrange and chromosome enumeration probe10 (*CEP10*) SpectrumGreen probes (Vysis Inc., Downers Grove, IL, USA) were used according to manufacturer instruction. Briefly, deparaffinized sections were heated by microwave in 0.01 M citrate buffer (pH 6.0) and pretreated with 0.3% pepsin. Subsequently, the slides were immersed in 0.1% NP-

40 and denatured by heating in 70% formamide/2×SSC. The probe mixture was also denatured and applied to the pretreated tissues. The slides were incubated for 16 h at 37°C in a humidified chamber, then were washed, counterstained with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Vysis Inc.), and photographed. Signals were analyzed in 10 viewing areas per case at ×1000 magnification to calculate the average *PTEN*/*CEP10* ratio.

Droplet Digital PCR (DDPCR) for *PTEN*

ddPCR was performed to confirm *PTEN* genomic copy number for comparison with the FISH results. Genomic DNA was extracted from tumor and normal areas in tissues as described [17]. Tumor areas as identified by a guide slide stained with hematoxylin and eosin were microdissected from each 10-μm-thick section and transferred into tubes. Paraffin removal was performed in 80% xylene; then tissues were washed twice with absolute ethanol and deparaffinized tissue pieces were spun down at 15,000 × *g* for 10 min. After drying, the pellets were resuspended in 360 μl buffer ATL (QIAamp DNA Mini Kit, Qiagen, Hilden, Germany) and incubated at 95°C for 15 min, then cooled to room temperature. Samples were immediately digested with proteinase K for 72 h at 56°C in a rotation oven with periodic mixing and addition of fresh proteinase K every 24 h. DNA was collected using the QIAamp DNA Mini Kit according to manufacturer instruction. Extracted DNA was quantified on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and 50 ng DNA was used for ddPCR according to manufacturer protocol (Bio-Rad Laboratories, Inc., Pleasanton, CA, USA). The following primers and TaqMan® probes were used for ddPCR. *PTEN* DNA: forward: 5′-CTATTCCAATGTTCAGTGG-3′, reverse: 5′-GTTCCAATACATGGAAGGAT -3′, probe: 5′-FAM-CAAGATGATGTTTGAAACTA-3′; Vimentin DNA forward: 5′-AAGTGTGGCTGCCAAGAACCT-3′, reverse: 5′- CTTTGGTTGAAGCCGCACTGA-3′, probe: 5′-VIC-

ACAAATCCAAGGTAGGAAA-3′. Thermal cycling conditions were 95°C for 10 min; 40 cycles of 94°C for 30 s, 60°C for 60 s, 98°C for 10 min, and a 12°C hold. The *PTEN*/Vimentin ratio was calculated. Vimentin was used as an endogenous control.

Statistical Analyses

The effect of grade on the prognosis of NENs in our series was measured as a hazard ratio (HR) with 95% confidence interval (CI) using a multivariate Cox proportional hazards model. The Cochran-Armitage test was used to compare the incidence of NE marker and p53 immunoreactivities and of Ki-67 LI among tumor grades (G1, G2, or G3). Associations between the SSTR2A expression score or the level of PTEN immunoreactivity and tumor grades were assessed by the Jonckheere-Terpstra test. The Fisher's exact test was used to compare the incidence of NE markers and p53 immunoreactivities and of Ki-67 LI among G3 organs. Associations between SSTR2A expression score or PTEN immunoreactivity levels and G3 organs were assessed by the Kruskal-Wallis test. Associations between p53 expression and SSTR2A expression score or the PTEN immunoreactivity levels were assessed by the Cochran-Armitage test, and between SSTR2A expression score and PTEN immunoreactivity level was assessed by the Jonckheere-Terpstra test. Furthermore, associations between PTEN immunoreactivity level and FISH or ddPCR *PTEN* gene copy number results in NENs were assessed by the Jonckheere-Terpstra test. Correlation between FISH and ddPCR analyses results were evaluated by Pearson's correlation analysis. The PHREG procedure in SAS software (version 8.2; SAS Institute, Cary, NC, USA) was used for calculations. All tests were one-tailed, and a p-value <0.05 was considered statistically significant.

Results

Overall Survival (OS) Rate of NENs Based on Grading According to GEP-NET 2010 WHO Classification

Among a total of 99 NEN cases, 59 could be confirmed for OS at 36 months after tumor resection. OS based on NEN grade is summarized in Table 2 along with other patient profile information. Our statistical analysis revealed that the OS rate of NENs was significantly poorer at the higher grade (G3) (HR: 12.4, 95% CI: 2.3–65.7).

Immunohistochemistry for NE Markers/P53/SSTR2A/PTEN Expression and Ki-67 LI

Immunohistochemical results based on NEN grade are summarized in Table 3. Although a high incidence of NSE expression was significantly ($p = 0.0363$) associated with lower NEN grade, no significant differences existed between CgA, Syn, and CD56 incidence and NEN grade. Both Ki-67 LI and p53 expression were significantly ($p < 0.0001$) higher in higher NEN grades. Conversely, both SSTR2A expression scores and PTEN immunoreactivity levels were significantly ($p < 0.0001$) lower in higher NEN grades. Statistical analyses of the relationships between tumor-related molecule expression in NENs revealed a significant negative association between p53 and SSTR2A ($Z = -2.2109$, $p = 0.027$) or PTEN ($Z = -3.1512$, $p = 0.0016$) and a significant positive association between SSTR2A and PTEN ($Z = +4.3890$, $p < 0.0001$). Representative images of tumor-related expression in NENs are depicted in Fig. 2.

To clarify the difference in p53, SSTR2A, and PTEN expression among sites of NEN origin, we used 46 cases of G3, consisting of 10 GEP, 31 lung, and 5 other. The immunohistochemical results based on sites of G3 origin are summarized in Table 4. For NE marker expression in G3, although CD56-positivity was significantly ($p = 0.0329$) higher in tumors of lung origin than other organs, there were no significant differences between CgA, Syn, and NSE expression or between Ki-67 LI/p53 expression/SSTR2A score/PTEN immunoreactivity and G3 origin site.

FISH and DDPCR Analysis for *PTEN* Gene Copy Number and Comparison with PTEN Immunoreactivity in NEN

Only total 22 cases were available for both FISH and ddPCR analyses because of limitations in the quality and quantity of the DNA extracted from FFPE samples. FISH and ddPCR analyses results are summarized in Table 5 along with NEN grade and site of NEN origin. Representative images of PTEN immunoreactivity and the corresponding FISH signals in NENs are presented in Fig. 3. Our statistical analyses revealed that the level of PTEN immunoreactivity was significantly ($p = 0.0365$) associated with the results of both FISH and ddPCR analysis and a significant correlation ($r = 0.64$, $p = 0.0013$) was identified between FISH and ddPCR analyses results for NEN *PTEN* gene copy number.

Discussion

NENs occurring in various organs share histological findings but have been assigned distinct diagnostic criteria (such as mitotic level and NE marker immunohistochemical properties) and varying treatment options, which might induce a diagnostic discrepancy among histologically identical tumors. This study aimed to clarify whether NENs of different organs are comparable at the molecular pathologic level and in particular, for the expression of SSTR2A and PTEN, which can be predictors of sensitivity to somatostatin analogue and mTOR inhibitor treatment, respectively. This study also aimed to analyze the associations between tumor-related molecule expression and NEN grade and between the level of PTEN immunoreactivity and its FISH or ddPCR results to verify the significance of immunohistochemistry as a surrogate for genome copy number.

In our series of GEP-NENs, the proportions of G1/G2/G3 were 63.3%/16.3%/20.4%, respectively. These are very comparable to the results of other reports that classify their cases as proportions of G1/G2/G3; these are 64.9–

73.2%/6.3–10.5%/16.3–24.7%, respectively [18–20]. The most common type of lung NEN is reported to be SCC, which accounts for 60%–80% of pulmonary NENs and, together with another highly malignant NEN, LCNEC, represents over 80%; in comparison, two lung NENs with low- to intermediate-grade malignancy (typical and atypical carcinoids) represent less than 20% [21–23]. In our lung NEN cases, the proportions of G1/G2/G3 were 10.8%/5.4%/83.8%, indicating a concordance with the previous data. Thus, our histological classification by a single grading system appears to have been implemented correctly.

By employing a single grading system to multi-organ NENs regardless of tumor location, we revealed that no significant differences existed in the immunohistochemical profiles of NE markers, p53, SSTR2A, or PTEN expression in G3 tumors among sites of origin. Furthermore, consistent with previous reports [24–29], both Ki-67 LI and p53 expression significantly increased with NEN grade, suggesting a carcinogenic role of p53 mutations in NENs with mitotic activity. Conversely, SSTR2A and PTEN expression levels significantly decreased with tumor grade progression. Negative associations between both SSTR2A/PTEN and p53 levels and positive association between the SSTR2A and PTEN levels were also demonstrated, suggesting that alteration in two major tumor suppressor genes was involved in the etiology of highly malignant or late-stage NEN. Furthermore, loss of membranous SSTR2A expression appeared to be a common event in G3 regardless of tumor origin and was associated with poor-differentiation of NENs via PTEN and p53 mutation, likely because SSTR2A acts as a mediator of neuroendocrine functions such as neurotransmission, hormone secretion inhibition, immune system regulation, and cell proliferation inhibition [30].

PTEN plays an essential role as a negative regulator in the PI3K-AKT-mTOR pathway, which has been found to be somatically deleted, mutated, and/or silenced in various sporadically occurring cancers such as glioblastoma, malignant melanoma, and thyroid, breast, endometrial, and ovarian carcinomas [31–37]. Functional *PTEN* inactivation might

occur through deletion, LOH, or other mutations [6, 38]. In sporadic pancreatic NEN, somatic *PTEN* mutations including indels and missense mutations have been found in 7.3% of cases by whole exomic sequencing [39]. In small cell lung cancers, loss of *PTEN* has been identified in 75.8% of cases by array comparative genomic hybridization methods [40]. Thus, *PTEN* inactivation is suggested to be involved in NEN carcinogenesis at various sites. The present study demonstrated that PTEN immunoreactivity in NENs was significantly associated with genomic copy number by FISH analyses and verified by ddPCR methods. Thus, immunohistochemical determination of PTEN expression level might serve as a surrogate for *PTEN* genomic copy number.

NENs are thought to consist of a diverse group of neoplasms in terms of origin, mechanism of development, functional status, histologic patterns, and biological behavior, indicating an underlying heterogeneity. Since the grading and staging schemes are still evolving, it is expected that the grade definition might need future adjustment, following the accumulation of additional follow-up or molecular data. In this study, we aimed to demonstrate from a pathological point of view that a single grading system is applicable to multi-organ NENs regardless of tumor site. Indeed, this study provides evidence that the GEP-NET 2010 WHO classification might be useful to predict the prognoses of patients with multi-organ NENs and that the expression of the SSTR2A and PTEN mTOR-associated proteins universally indicates tumor aggressiveness/grade.

In conclusion, the present study revealed no significant differences in immunohistochemical profiles of NE markers, p53, SSTR2A, or PTEN expression in NENs among sites of origin, whereas PTEN and p53 functional inactivation and loss of membranous SSTR2A expression appears to be commonly involved in the etiology of highly malignant NEN. Furthermore, immunohistochemical analysis of PTEN expression is suggested to act as a surrogate for *PTEN* genomic copy number. The current study will likely contribute to the expansion of biotherapeutic options such as

the use of mTOR inhibitors and SSTR2A analogues against NENs in various organs.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Table 1 Clinicopathologic profiles of tissue samples used in this study

Site of origin	*n*	M/F	Mean age (range, years)	Grade (%)			Sites in detail (*n*:%)
				G1	G2	G3	
GEP	49	32/17	61.4 (14–85)	31 (63.3)	8 (16.3)	10 (20.4)	Pancreas (4:8.2), Stomach (9:18.4), Small intestine (7:14.3), Colorectum (29:59.2)
Lung	37	34/3	69.1 (24–81)	4 (10.8)	2 (5.4)	31 (83.8)	
Other	13	10/3	60.3 (39–86)	2 (15.4)	6 (46.1)	5 (38.5)	Thymus (5:38.5), Mediastinum (3:23.1), Bladder (2:15.4), Breast (2:15.4), Prostate (1:7.7)
Total	99	76/23	64.2 (14–86)	37 (37.4)	16 (16.2)	46 (46.5)	

GEP: gastro-entero-pancreas

Table 2 Comparison of overall survival rate at 36 months after resection based on grade of neuroendocrine neoplasia

Grade	*n*	M/F	Mean age (range, years)	GEP/Lung/Other	Overall survival (rate, %)
G1	21	13/8	56.2 (24–72)	19/1/1	21(100)
G2	8	5/3	61.6 (48–70)	3/1/4	6 (75.0)
G3	30	27/3	71.0 (58–86)	6/20/4	13 (43.3)
Total	59	45/14	64.5 (24–86)	28/22/9	40 (67.8)

Table 3 Immunohistochemical results based on grade of neuroendocrine neoplasia

Grade	*n*	Neuroendocrine marker [n, (%)]				Ki-67 LI	P53	SSTR2A score				PTEN		
		CgA	Syn	CD56	NSE			0	1	2	3	Negative	Focal	Diffuse
G1	37	19 (51.4)	28 (75.7)	26 (70.3)	16 (43.2)	1	8 (21.6)	2 (5.4)	0	9 (24.3)	26 (70.3)	1 (2.7)	7 (18.9)	29 (78.4)
G2	16	12 (75.0)	15 (93.8)	14 (87.5)	5 (13.5)	6.9	8 (50.0)	4 (25.0)	1 (6.3)	4 (25.0)	7 (43.7)	0	9 (56.3)	7 (43.7)
G3	46	15 (32.6)	32 (69.6)	35 (76.1)	10 (21.7)	54.2	35 (76.1)	8 (17.4)	20 (43.5)	12 (26.1)	6 (13.0)	22 (47.8)	19 (41.3)	5 (10.9)
p-value		0.0704†	0.4682†	0.5744†	0.0363†	< 0.0001†	< 0.0001†	< 0.0001‡				< 0.0001‡		

†: assessed by the Cochran-Armitage trend test

‡: assessed by the Jonckheere-Terpstra test

PTEN: phosphatase and tensin homolog; SSTR2A: somatostatin receptor type 2A; CgA: chromogranin A; Syn: synaptophysin; NSE: neuron specific enolase; LI: labeling index

Table 4 Immunohistochemical results based on neuroendocrine carcinoma (G3) site

Site	n	Neuroendocrine markers [*n* (%)]				Ki-67 LI	P53	SSTR2A score				PTEN		
		CgA	Syn	CD56	NSE			0	1	2	3	Negative	Focal	Diffuse
GEP	10	4 (40.0)	9 (90.0)	5 (50.0)	2 (20.0)	55.4	8 (80.0)	1 (10.0)	5 (50.0)	4 (40.0)	0	7 (70.0)	2 (20.0)	1 (10.0)
Lung	31	8 (25.8)	21 (67.7)	27 (87.1)	6 (19.4)	57.7	23 (74.2)	5 (16.1)	13 (42.0)	8 (25.8)	5 (16.1)	12 (38.7)	15 (48.4)	4 (12.9)
Other	5	3 (60.0)	2 (40.0)	3 (60.0)	2 (40.0)	50.0	4 (80.0)	2 (40.0)	2 (40.0)	0	1 (20.0)	3 (60.0)	2 (40.0)	0
p-value		0.2439†	0.1054†	0.0329†	0.5626†	0.5979†	1.0000†	0.5679‡				0.2355‡		

†: assessed by Fisher's exact test

‡: assessed by Kruskal-Wallis test

GEP: gastro-entero-pancreas; PTEN: phosphatase and tensin homolog, SSTR2A: somatostatin receptor type 2A, CgA: chromogranin A, Syn: synaptophysin, NSE: neuron specific enolase, LI: labeling index

Table 5 Comparison of PTEN expression in neuroendocrine neoplasias by immunohistochemistry (IHC) with detection by fluorescence *in situ* hybridization (FISH) and droplet digital PCR (ddPCR)

Case	Site of origin	Grade	IHC	*PTEN/CEP10* by FISH	*PTEN*/vimentin by ddPCR
1	Lung	G3	Negative	0.64	0.7
2	Lung	G3		0.69	1.4
3	Lung	G3		0.08	0.3
4	Pancreas	G1	Focal	0.91	1.1
5	Intestine	G2		0.53	0.6
6	Colon	G3		0.81	0.8
7	Lung	G2		0.8	0.8
8	Lung	G3		0.09	0.4
9	Lung	G3		0.77	1.3
10	Lung	G3		0.27	0.8
11	Lung	G3		0.15	0.8
12	Lung	G3		0.47	0.7
13	Lung	G3		1	1.7
14	Lung	G3		0.17	1
15	Breast	G3		0.69	1
16	Stomach	G1	Diffuse	0.37	0.8
17	Stomach	G2		1.03	1.5
18	Intestine	G1		1.05	0.9
19	Rectum	G1		0.54	1
20	Lung	G3		0.65	1
21	Lung	G3		1.01	1
22	Mediastinum	G2		1.21	1.1

p-value assessed by Jonckheere-Terpstra test for association between IHC and FISH was 0.0365

p-value assessed by Jonckheere-Terpstra test for association between IHC and ddPCR was 0.1318

Correlation coefficient between FISH and ddPCR was 0.64 ($p = 0.0013$ by Pearson's correlation analysis)

Figure legends

Fig. 1 Representative images with Ki-67 labeling index of neuroendocrine tumor (NET) G1, G2, and G3/neuroendocrine carcinomas according to the grading system of the gastro-entero-pancreatic-NET 2010 WHO classification.

Fig. 2. Expression profiles of tumor-related molecules in NENs. **a**-**d** represent gastric G1 showing a 2% Ki-67 labeling index (LI), and **e**-**h** are pulmonary G3 showing 45% Ki-67 LI. **b**/**f**, **c**/**g**, and **d**/**h** show the immunoreactivities of p53, somatostatin receptor type 2A (SSTR2A), and phosphatase and tensin homolog (PTEN) expression, respectively. Original magnification: ×200.

Fig. 3 Comparison of the level of phosphatase and tensin homolog (PTEN) immunoreactivity (**a**-**c**) and its corresponding fluorescence *in situ* hybridization (FISH) signals (**d**-**f**) in neuroendocrine neoplasia. **a**/**d**, **b**/**e**, and **c**/**f** are from case 3, case 12, and case 21 in Table 4, respectively. The *PTEN* signals are labeled using SpectrumOrange and chromosome enumeration probe10 (CEP10) are labeled with SpectrumGreen. Original magnifications: **a**-**c**, ×40; **d**-**f**, ×1000.

Figure 1

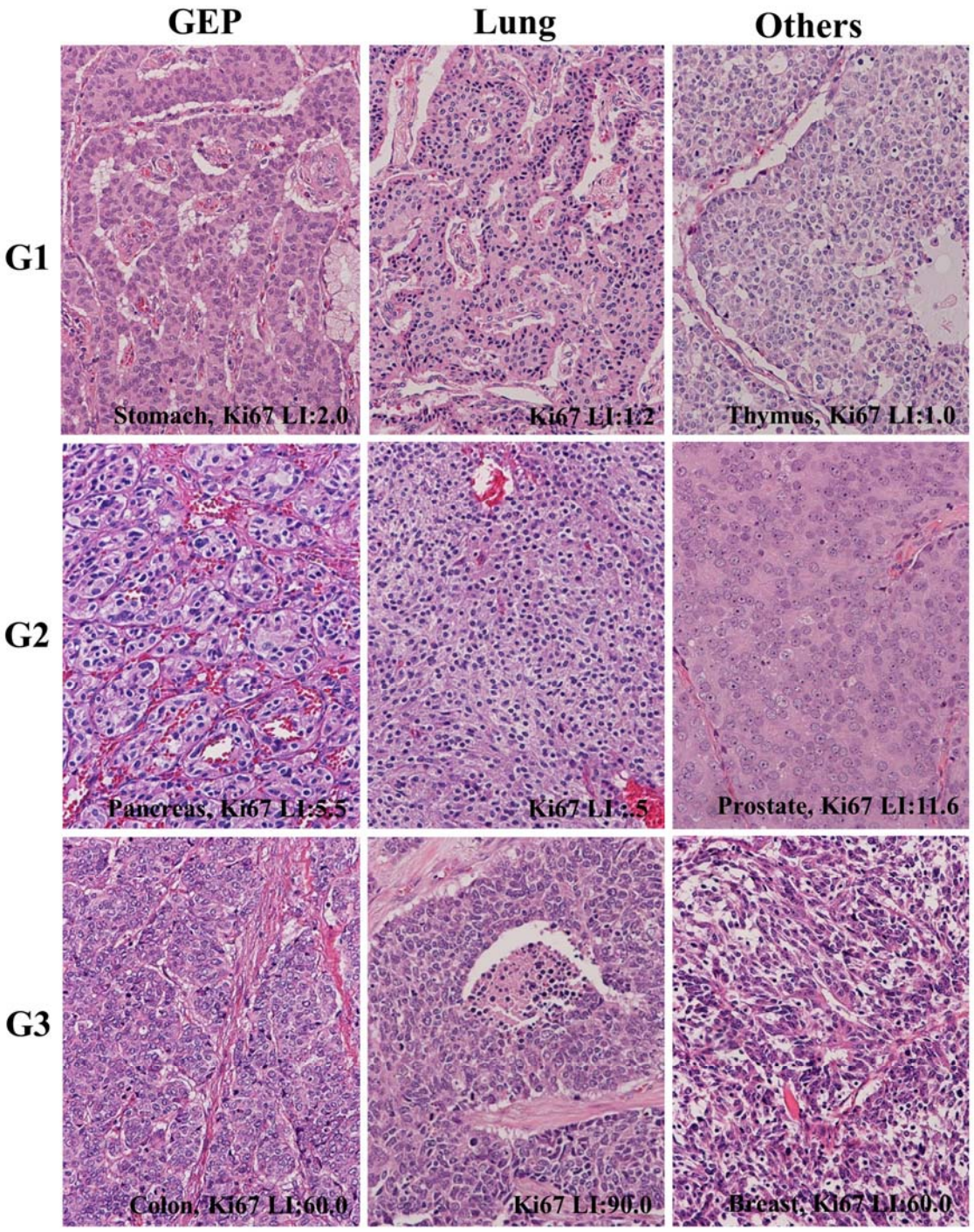
GEP
Lung
Others
G1
G2
G3
Stomach, Ki67 LI:2.0
Ki67 LI:1.2
Thymus, Ki67 LI:1.0
Pancreas, Ki67 LI:5.5
Ki67 LI:.5
Prostate, Ki67 LI:11.6
Colon, Ki67 LI:60.0
Ki67 LI:90.0
Breast, Ki67 LI:60.0

Figure 2

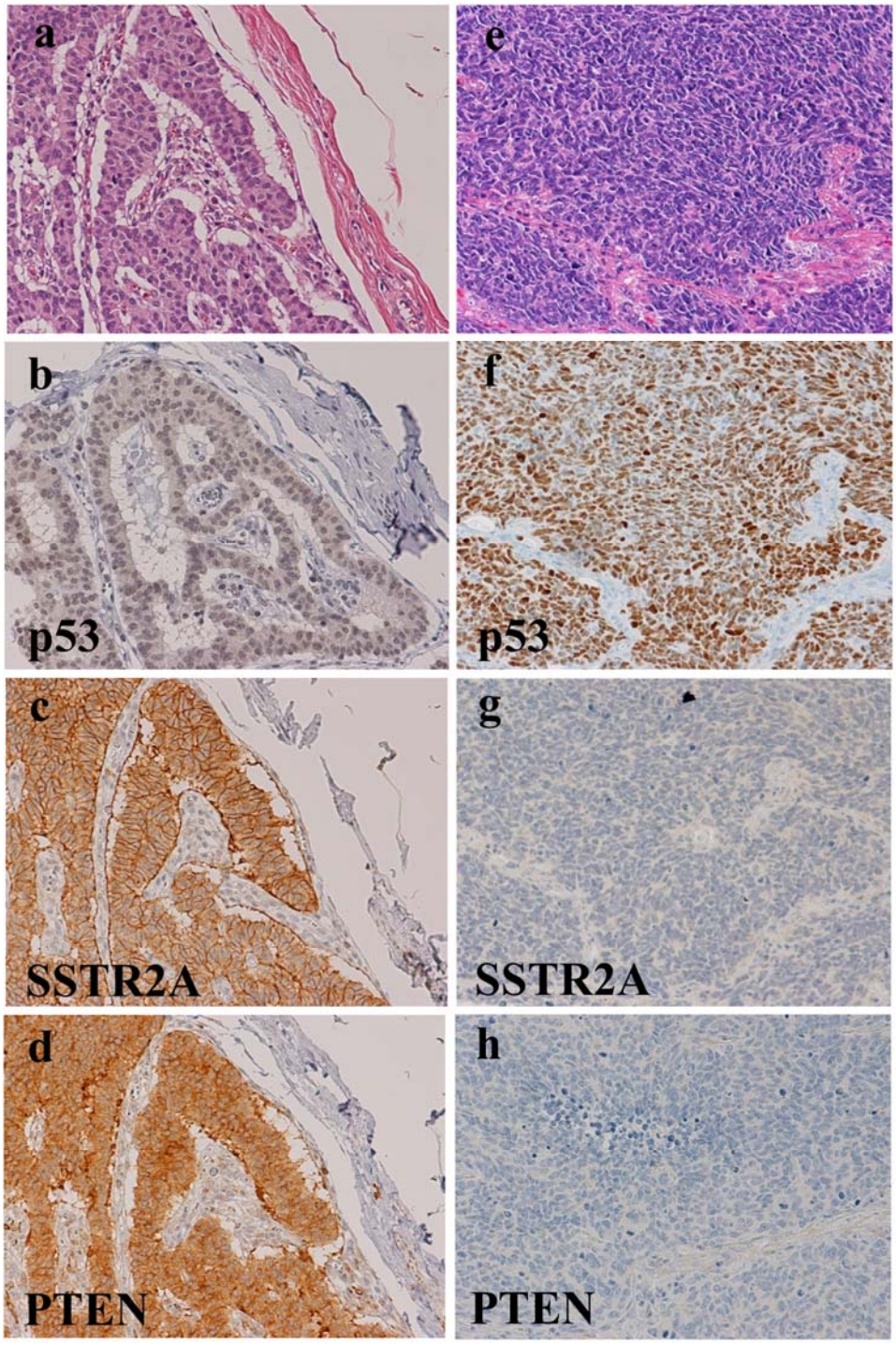

Figure 3