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Alterations in Cancer-related Genes Associated with Grading of Well Differentiated Pancreatic Neuroendocrine Neoplasms

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Abstract

Objectives: Although recent advances in next-generation sequencing (NGS) have revealed some genetic alterations in various tumors, including pancreatic neuroendocrine tumors (PanNETs), their clinical significance is not fully understood. To investigate the clinical significance of gene alteration in PanNETs, we performed genetic analysis of well differentiated PanNETs using NGS.

Methods: Twenty-nine resected primary PanNET tissue samples and three samples of metastatic liver tissues, obtained from 29 PanNET patients, were analyzed. DNA was extracted from laser-captured formalin-fixed paraffin-embedded tissues, and 50 cancer-associated genes, including approximately 2,800 hotspots, were amplified by multiplex PCR. Amplified libraries were sequenced using NGS, and the results were analyzed in conjunction with respective clinicopathological features.

Results: Among 50 investigated genes, somatic mutations were observed in four of 29 PanNET cases. We identified APC mutations in three cases, PTEN in two, and VHL and STK11 in one. The identified mutations were observed only in NET G2 tumors. All liver metastases contained at least one mutation, such as PTEN or TP53, which was not observed in the primary tumor.

Conclusion: The cancer-related gene mutations observed in PanNETs were associated with G2 grade tumors. The mutations were more frequent in PanNET liver metastasis than in the primary tumors. Our analysis of liver metastasis cases suggested that cancer-related gene mutations might raise the tumor grade and promote liver metastasis. Further studies of associations between genetic alterations and clinicopathological features should help in the cancer diagnosis and prediction of therapeutic effects of molecular-target drugs.

Keywords: Pancreatic neuroendocrine tumor; WHO 2010 grading classification; Mutation; Next-generation sequencing

Introduction

Pancreatic neuroendocrine tumors (PanNETs) are rare tumors that comprise 1–2% of pancreatic neoplasms [1], with an annual incidence of 0.32/100,000 in the United States [2] and 1.27/100,000 in Japan [3]. Most of these tumors are more indolent than other epithelial malignant tumors; however, they can be aggressive, with the 5-year survival of 27-79% [2,4]. Surgery is the only curative treatment for PanNETs in the absence of metastasis to other organs. Although there is evidence to support hepatectomy for resectable liver metastases [5], the effectiveness of resection of the primary tumor in the presence of unresectable liver metastases remains controversial [6,7]. In such cases, other therapies might be used, including somatostatin analogs, peptide-receptor radionuclide therapy, liver-directed chemoembolization, radiofrequency ablation of hepatic metastases, and chemotherapy [8]. Recently, molecular-targeted agents such as sunitinib or everolimus have been used as a first-line therapy for patients with advanced and Grade 1 (G1) or G2 tumor (World Health Organization (WHO) classification 2010) [9,10].

Although prognosis evaluation for patients with these tumors is difficult, recent studies have established TNM staging and grading as independent prognostic factors of PanNETs [11,12]. The European Neuroendocrine Tumors Society has proposed a 3-tier grading classification system based on the mitotic rate and/or the Ki-67 proliferative index [13]. The subsequently published classifications of the Union for International Cancer Control and WHO were based on the classification by European Neuroendocrine Tumors Society [14]. Recent advances in endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) have enabled preoperative grading of PanNETs [15]. These classifications are important to determine the indications for chemotherapy and molecular-targeted agents [9,10].

Majority of PanNETs occur as sporadic, isolated tumors, although some PanNETs are a part of complex familial endocrine cancer syndromes such as multiple endocrine neoplasm type 1 (MEN-1), MEN-2, von Hippel–Lindau disease (VHL), neurofibromatosis type 1, and tuberous sclerosis complex [8]. Recent whole-exome sequencing identified several frequently mutations in sporadic well-differentiated

PanNETs, including in MEN1, DAXX, ATRX, PTEN, TSC2, and PIK3CA [16]. Some of these genes are tumor suppressor genes but the role of these genetic alterations in tumorigenesis and progression of PanNETs and their clinical significance remain unclear.

Recent advances in next-generation sequencing (NGS) technology allow a rapid and inexpensive identification of actionable mutations in multiple genes. DNA isolated from formalin-fixed paraffin embedded (FFPE) clinical tissue specimens is often of poor quality and available in very limited quantities; however, sufficient sequencing libraries can be obtained by multiplex PCR with short amplicons [17]. In this study, we analyzed genetic alterations in the FFPE samples of resected well differentiated PanNETs (NET G1/G2) using NGS technology to clarify the clinicopathological significance of genetic mutations in PanNETs.

Subjects and Methods

Patients

We used tumor tissue samples from 29 patients whose PanNETs were surgically resected between 1990 and 2013 at Yamanashi University Hospital. Clinical information, including age, sex, symptoms, tumor size, localization at presentation, hereditary status, pathology, TNM stage, surgical procedure, and follow-up time, were retrospectively collected from the medical records. All tumors were histologically reviewed and classified into three grades according to the WHO 2010 classification [14]. This study was approved by the Human Ethics Review Committee of Yamanashi University Hospital.

DNA extraction

Tumor tissue and adjacent normal pancreatic tissue were separately laser-microdissected from the same 8-μm-thick sections of FFPE blocks using an Applied Biosystems ArcturusXT microdissection instrument (Life Technologies, Foster City, CA). DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Milan, Italy) following the manufacturer's instructions. Extracted DNA was quantitated by measuring the optical density at A260-nm and A280-nm wavelengths.

Multiplex PCR of targeted genes and deep sequencing

Amplification of targeted genes and deep sequencing were performed as described previously [18]. In brief, 10-ng aliquots of extracted DNA were amplified using multiplex PCR methods with the ready-made premixed primer kit (Ion AmpliSeq Cancer Hotspot Panel v2, Life Technologies). Approximately 2,800 mutations were targeted in the following 50 cancer-associated genes in the COSMIC database [19]: ABL1, AKT1, ALK, APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4, EZH2, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAS, GNAQ, HNF1A, HRAS, IDH1, JAK2, JAK3, IDH2, KDR/VEGFR2, KIT, KRAS, MET, MLH1, MPL, NOTCH1, NPM1, NRAS, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SMO, SRC, STK11, TP53, and VHL. The primer kit contains 207 primer pairs with a relatively short amplicon length to improve the PCR performance impaired by the fragmentation of DNA extracted from FFPE. The PCR products were checked using a high-sensitivity DNA assay on the Agilent 2100 Bioanalyzer on-chip (Agilent Technologies, Santa Clara, CA, USA).

The PCR products were further amplified by emulsion PCR using OneTouch OT2 system (Life Technologies) and then sequenced on the Ion Torrent PGM sequencer (Life Technologies) loaded with a 318 Chip. Torrent Suite Software v.4.0 (Life Technologies) was used to align reads to the hg19 reference genome and generate run metrics, including total read counts and quality. Variant Caller v.4.0 (Life Technologies) was used to identify variants. When the variants were detected in both tumor tissue and normal pancreatic tissue, they were considered germ-line variants. Variants identified only in tumor tissue and identical to the COSMIC database were considered tumor-specific somatic mutations, and the cut-off value of variant frequency per read depth was set at 8% to avoid erroneous variant calls [17].

Immunohistochemistry

Immunohistochemistry was performed as reported previously [20]. In brief, deparaffinized sections of formalin-fixed tissue of 2-μm thicknesses were stained with primary antibodies specific for Ki-67 (1:100 dilutions; cat, M7240; Dako, Carpinteria, CA, USA). Antigen retrieval was achieved by boiling the tissue sections in Target Retrieval Solution (Dako) in an autoclave unit for 20 min at 120°C before incubation with anti-Ki-67. Envision kit/HRP (AEC) (cat. K1491; Dako) was used as the secondary antibody, and diaminobenzidine was used as the chromogen. All sections were counterstained with hematoxylin. Following the WHO 2010 recommendations [14], the Ki-67 index was calculated by counting 2000 cells in areas of the strongest nuclear labeling to establish the grade of each PanNET.

Statistical Analysis

Because the tumor grade has prognostic significance for PanNET, its association with genomic alterations and clinicopathological variables was assessed. Continuous variables were compared using Mann–Whitney U-test. Categorical variables were compared using $\chi 2$ or Fisher's exact test as appropriate. A p-value of <0.05 was considered statistically significant.

Results

Patient characteristics

A summary of the clinicopathological features of the 29 PanNETs is shown in Table 1. There were five functioning tumors, including three insulinomas and two gastrinomas. We observed two patients with inherited PanNET syndromes and both had MEN1. One of them had a pancreatic gastrinoma, and it was diagnosed as MEN1 because of the familial history of hyperparathyroidism and pituitary microadenoma.

The other patient had multiple non-functioning PanNETs with liver metastasis, and the cancer was diagnosed as MEN1 because of the multiple lesions and hyperparathyroidism. Among the 29 patients, 18 (62.0%) had NET G1 and 11 (37.9%) had NET G2. There were no cases of neuroendocrine carcinoma or mixed adenoneuroendocrine carcinoma. Three patients had distant metastases, two of whom had metastasis in the liver and the third had both liver and nodal involvement. During the mean follow-up period of 799 days (range, 30–5,177 days), two patients died because of liver metastasis progression.

Characteristic	Value
Age (years), median (range)	61 (33-81)
Sex	
Female	13
Male	16
Symptoms	
Symptomatic	4
Abdominal pain	1
Hypoglycemia	3
Asymptomatic	25
Functioning	
Yes	5
No	24
Size (mm), median (range)	12 (2-80)
Location	
Body or tail	18
Head	11
Tumor feature	
Cystic component	5
Encapsulated	6
Hereditary status	
Sporadic	27
MEN-1	2
WHO Grade	
NET G1	18
NET G2	11
UICC Stage	
I	20
II	5
III	1
IV	3
Surgical treatment	
Enucleation	3
Segmental resection	7
DP	9
PD	10

Follow-up (days), median (range)	799 (30-5117)
Outcome	
Alive	23
Dead	2
NA	4
DP: Distal Pancreatectomy; PD: Pancreatoduodenectomy; NA: Not Available.	

Table 1: Baseline characteristics of the 29 tumors.

Deep sequence analysis of 50 cancer-related genes in resected tissues of primary PanNET

The mean DNA concentration of 25.8 ng/μl was obtained from a microdissected sample of approximately 7 mm^2. The average of 364,904 mapped sequence reads for each sample and 1,280 reads for each amplicon were achieved. The average of 19.4 variants was detected in each sample, of which 3.4 variants existed in the COSMIC database. We identified four mutated genes in four (13.8%) patients (Figure 1). APC was mutated in three patients (10.3%), PTEN in two (6.9%), STK11 in one (3.4%), and VHL in one patient (3.4%). One patient had mutations in three genes (APC, PTEN, and STK11), another patient in two (APC and VHL), and the remaining two patients had one mutation each, in APC and VHL. Mutations in APC, PTEN, and STK11 were frameshift mutations and the mutation in VHL was a missense. All identified mutations were observed in patients with NET G2, and there were no mutations in patients with NET G1 (Table 2).

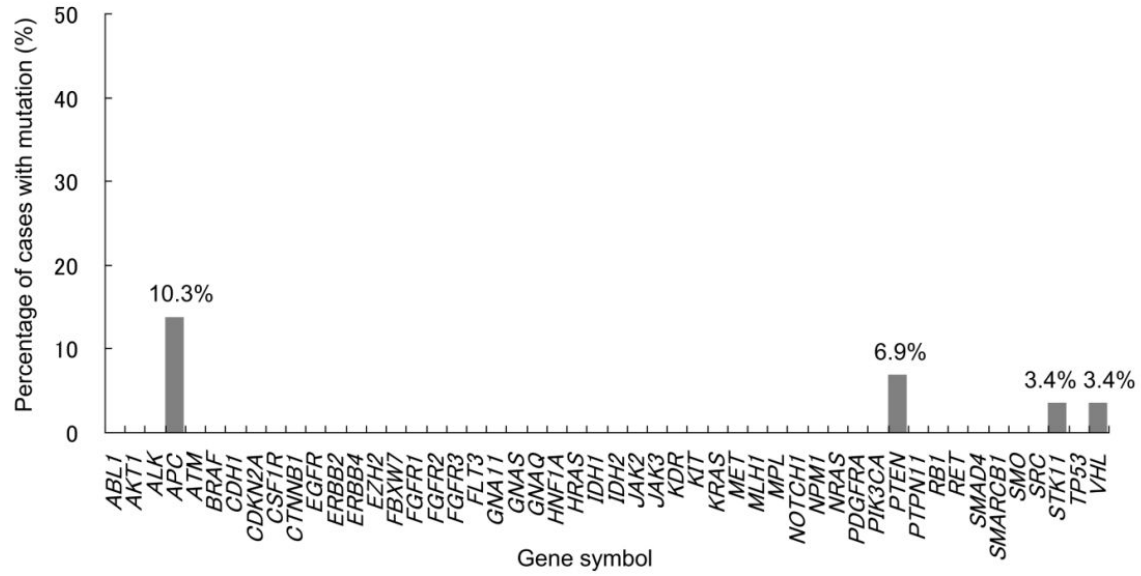

Figure 1: Percentage of cases with mutations in each of the 50 cancer-related genes. APC mutations were detected in 10.3% (three of 29 cases), PTEN mutations in 6.9% (two of 29 cases), both STK11 and VHL mutations in 3.4% (one of 29 cases).

Mutations											
	APC		**PTEN**		**STK11**		**VHL**				
	Amino acid	VF	Amino acid	VF	Amino acid	VF	Amino acid	VF	Tumor size		
Case	substitution	(%)	substitution	(%)	substitution	(%)	substitution	(%)	(mm)	Grade	Stage
1	p.N1455fs*18	17.6	p.T321fs*23	30.6	p.P281fs*6	28.6	WT		19	G2	I

2	p.T1556fs*9	18.1	WT		WT		WT		35	G2	III
3	p.T1556fs*9	18.1	WT		WT		p.P86S	86.7	80	G2	IV
4	WT		p.T321fs*23	33.3	WT		WT		10	G2	I
5	WT		WT		WT		WT		10	G2	I
6	WT		WT		WT		WT		30	G2	IV
7	WT		WT		WT		WT		13	G2	I
8	WT		WT		WT		WT		9	G2	I
9	WT		WT		WT		WT		18	G2	I
10	WT		WT		WT		WT		23	G2	II
11	WT		WT		WT		WT		9	G2	I
12	WT		WT		WT		WT		40	G1	II
13	WT		WT		WT		WT		12	G1	I
14	WT		WT		WT		WT		13	G1	I
15	WT		WT		WT		WT		9	G1	I
16	WT		WT		WT		WT		12	G1	I
17	WT		WT		WT		WT		15	G1	II
18	WT		WT		WT		WT		12	G1	I
19	WT		WT		WT		WT		30	G1	II
20	WT		WT		WT		WT		3	G1	I
21	WT		WT		WT		WT		12	G1	II
22	WT		WT		WT		WT		12	G1	I
23	WT		WT		WT		WT		14	G1	I
24	WT		WT		WT		WT		2	G1	I
25	WT		WT		WT		WT		8	G1	I
26	WT		WT		WT		WT		15	G1	I
27	WT		WT		WT		WT		5	G1	I
28	WT		WT		WT		WT		10	G1	IV
29	WT		WT		WT		WT		8	G1	I
PanNET: Pancreatic Neuroendocrine Tumor; VF: Variant Frequency; WT: Wild Type; fs: Frame Shift											

Table 2: Mutations and clinicopathological data of PanNET cases.

Association of clinical and mutational variables with tumor grades

The mutation status and other clinicopathological variables were statistically assessed for their association with the tumor grade (Table 3). Among these variables, only the presence of mutations in 50 cancer-related genes was significantly associated with NET G2.

Clinicopathological factors		NET G1	NET G2	P value
Age		63 (48-81)	56 (33-79)	0.393†

Sex	Female	9	4	0.702*
	Male	9	7	
Symptoms	Symptomatic	3	1	0.566*
	Asymptomatic	15	10	
Functioning	Yes	3	2	0.917*
	No	15	9	
Size		12 (2-40)	13 (9-80)	0.131†
Location	Body or tail	12	6	0.696*
	Head	6	5	
Cystic lesion	Yes	3	2	0.917*
	No	15	9	
Multiplicity	Solitary	15	11	0.268*
	Multicentric	3	0	
Lymphatic or bloodvessel invasion	Present	6	4	0.868*
	Absent	12	7	
Mutations	Present	0	4	0.014*
	No mutations	18	7	
Stage	I-III	17	9	0.539*
	IV	1	2	
PanNET: Pancreatic Neuroendocrine Tumor; †Mann-Whitney U test; *Fisher's exact test				

Table 3: Correlation of tumor grade with clinicopathological data of PanNET.

Deep sequence analysis of 50 cancer-related genes in patients with primary PanNET and liver metastasis.

Among the 29 patients, three underwent not only surgical resections of primary pancreatic tumors but also simultaneous liver resection of synchronous metastasis. We also analyzed these resected liver specimens for somatic mutations of the 50 cancer-related genes. Among the three liver specimens, one had a newly developed mutation in TP53 besides the same mutation in VHL as the primary pancreatic tumor, and the others had a newly developed mutation in PTEN or TP53. Two of three cases had an advanced tumor grade with a newly developed gene mutation (Table 4).

Primary				Liver Metastasis		
Case	**Grade**	**Mutations**	**VF (%)**	**Grade**	**Mutations**	**VF (%)**
	G2	APC	18.1	G3	TP53	96.9
		VHL	86.7		VHL	69.6
	G2	WT		G2	TP53	68.7
	G1	WT		G2	PTEN	65.3

WT: wild type; VF: variant frequency

Table 4: Mutations in the APC, VHL, and PTEN genes in patients with primary tumor and liver metastasis.

Discussion

Our understanding of the biological basis of PanNET and its clinical associations remains limited. We performed a comprehensive deep sequencing analysis of 50 cancer-related genes in resected well differentiated PanNET (NET G1/G2) tissues using NGS and analyzed the associations with their clinical variables. We identified mutations in APC (10.3%), PTEN (6.9%), VHL (3.4%), and STK11 (3.4%) exclusively in PanNET G2 tumors, which carry a higher malignancy grade than PanNET G1. The results of the pair analysis for primary tissue and metastasis to the liver showed that the liver metastatic tissues had a higher mutation frequency and were of the higher grade than the primary PanNET.

Recent advances in NGS have enabled rapid, cost-effective, and comprehensive gene sequencing, resulting in the accumulation of evidence of gene alterations in various tumors, including PanNET [16,17,21]. Two recent studies have reported comprehensive genetic analyses of PanNET cases. Jiao et al. have conducted whole-exome sequencing of PanNET and identified mutations in MEN1 (44.1%),

DAXX (25%), ATRX (17.6%), PTEN (7.3%), TSC (28.8%), and PIK3CA (1.4%) [16]. Young et al. have conducted NGS of 287 cancer-related genes for various neoplasms (including PanNET) and identified the STK11 mutation [22]. The previously reported genes MEN1, DAXX, ATRX, and TSC were not assessed in our study, because they were not included in the multiplex PCR kit that we used. APC, PTEN, VHL, and STK11, in which we detected mutations in G2 but not in G1 tumors, are all tumor suppressor genes. A reduction in the tumor suppression function might have raised the histological grade of PanNETs. APC is a member of the Wnt signaling cascade and protects β-catenin from dephosphorylation by PP2A, thereby enhancing β-catenin phosphorylation and degradation. The loss of APC function stabilizes β-catenin and promotes its nuclear translocation, resulting in cell cycle progression, and enhances the expression of oncogenes [23-25]. There are only two reports investigating APC mutation in PanNET; one study examined a clinical sample with no mutation and the other examined two PanNET cell lines with APC mutations [22,26]. However, there are seven reports that examined β-catenin abnormality, and only six (3.1%) of 191 reported cases have either nuclear accumulation of β-catenin or β-catenin mutations [27-33]. PTEN and STK11 suppress the activation of the mTOR signaling pathway, and loss-of-function mutations for these genes result in the activation of this signal [34,35]. Recently, the mTOR pathway inhibitors have been shown to increase progression-free survival in advanced cases of PanNETs [9]; the mutational status of the mTOR pathway genes may predict the clinical response to mTOR inhibitors. Our data showed a mutation of either PTEN or STK11 in two of 29 cases (6.9%), the rate equivalent to that observed in the previous exome-sequence study [16]. VHL is a tumor suppressor gene in VHL disease in which PanNETs are observed in 12% to 17% of cases [36]. Mutation of VHL causes disruption of the interaction between the transcription factor HIF and VHL protein, leading to constitutive HIF activation and expression of HIF targets, affecting many cellular processes such as angiogenesis and cell metabolism [37,38]. One case of a VHL mutation in our study could be considered sporadic because the mutation was observed only in the tumor and not in a non-tumor sample.

Our analysis of liver metastasis cases suggests that cancer-related gene mutations may raise the tumor grade and promote liver metastasis. Liver metastasis has been reported in more than 50% of cases of PanNET [39], and the elevation of the Ki-67 index in primary tumors increases the probability of metastasis [40]. The Ki-67 index or tumor grade is higher for liver metastatic sites than primary sites [40,41]; however, there are no reports examining the mutational status of both primary and metastatic sites. In our analysis of three PanNET patients with liver metastasis, all three metastatic site had a newly developed mutation, which was not detected in the primary site, and two of three cases had an advanced grade in the metastatic site than in the primary site. This suggests that the elevation of the tumor grade and presence of a mutation might promote liver metastasis.

In the future, it might be possible to assess the tumor grade and predict therapeutic effects of molecular-target drugs more precisely. Recent advances in the EUS-FNA technique make it easy to obtain tumor tissues preoperatively or before chemotherapy administration [42,43]. The concordance rate of the Ki-67 index of PanNETs between resected specimens and EUS-FNA specimens is reported to be 74% [15]. In addition to the elevation of the Ki-67 index, the cancer-related gene mutation in EUS-FNA specimens might indicate increased chances of being a G2 tumor. It might be possible to predict therapeutic effects of molecular-targeted agents such as mTOR inhibitors using EUS-FNA specimens for the detection of mutations in the mTOR pathway, such as PTEN, STK11, and PIK3CA. Our analysis and another study detected mutations in the mTOR pathway in 6.9–15.0% of PanNET cases [16]. Moreover, multiple gene analysis of clinically obtained specimens such as EUS-FNA and pancreatic juice samples is already possible [18,22].

For limitations, the number of patients included in our study may be small for adequate statistical analysis, and our study observed mutations of cancer-related genes only in patients with G2 tumors and demonstrated a high incidence of mutations in metastatic liver sites. However, there were no patients with more malignant G3 tumors and only three patients with liver metastasis were observed in our study. Some of the genes whose alterations have been previously reported were not analyzed here because they were not included in the ready-made panel that we used. Comprehensive gene analysis in such malignant tumors would give us more clinically important findings.

In conclusion, cancer-related gene mutations were observed in G2 PanNETs using NGS analysis of resected well differentiated PanNET (NET G1/G2) samples. Such mutations and a higher grade of cancer were observed in some of the liver metastatic sites, whereas no mutations were detected in the respective primary sites. These findings may pave the way to improvements in cancer diagnosis, prognosis, and treatment, especially in combination with some new techniques such as EUS-FNA.

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