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Rb Loss and *KRAS* Mutation Are Predictors of the Response to Platinum-Based Chemotherapy in Pancreatic Neuroendocrine Neoplasm with Grade 3: A Japanese Multicenter Pancreatic NEN-G3 Study

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Abstract

Purpose: Patients with pancreatic neuroendocrine neoplasm grade-3 (PanNEN-G3) show variable responses to platinum-based chemotherapy. Recent studies indicated that PanNEN-G3 includes well-differentiated neuroendocrine tumor with G3 (NET-G3). Here, we examined the clinicopathologic and molecular features of PanNEN-G3 and assessed the responsiveness to chemotherapy and survival.

Experimental Design: A total of 100 patients with PanNEN-G3 were collected from 31 institutions, and after central review characteristics of each histologic subtype [NET-G3 vs. pancreatic neuroendocrine carcinoma (NEC-G3)] were analyzed, including clinical, radiological, and molecular features. Factors that correlate with response to chemotherapy and survival were assessed.

Results: Seventy patients analyzed included 21 NETs-G3 (30%) and 49 NECs-G3 (70%). NET-G3 showed lower Ki67-labeling index (LI; median 28.5%), no abnormal Rb expression (0%), and no mutated *KRAS* (0%), whereas NEC-G3 showed higher Ki67-LI (median 80.0%), Rb loss (54.5%), and *KRAS* mutations (48.7%). Chemotherapy response rate (RR), platinum-based chemotherapy RR, and prognosis differed significantly between NET-G3 and NEC-G3. Chemotherapeutic outcomes were worse in NET-G3 ($P < 0.001$). When we stratified PanNEN-G3 with Rb and *KRAS*, PanNENs-G3 with Rb loss and those with mutated *KRAS* showed significantly higher RRs to platinum-based chemotherapy than those without (Rb loss, 80% vs. normal Rb, 24%, $P = 0.006$; mutated *KRAS*, 77% versus wild type, 23%, $P = 0.023$). Rb was a predictive marker of response to platinum-based chemotherapy even in NEC-G3 ($P = 0.035$).

Conclusions: NET-G3 and NEC-G3 showed distinct clinicopathologic characteristics. Notably, NET-G3 does not respond to platinum-based chemotherapy. Rb and *KRAS* are promising predictors of response to platinum-based chemotherapy for PanNEN-G3, and Rb for NEC-G3. *Clin Cancer Res; 23(16); 4625–32. ©2017 AACR.*

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Translational Relevance

Emerging evidence indicates that pancreatic neuroendocrine neoplasm grade-3 (PanNEN-G3) defined by the WHO 2010 classification contains not only poorly differentiated neuroendocrine carcinoma (NEC-G3) but a well-differentiated type (NET-G3). Response of PanNEN-G3 to chemotherapy varies greatly among cases, but patient stratification was not fully clarified. This multicenter, retrospective study was one of the largest investigations including 70 patients with PanNEN-G3. After demonstrating two distinct categories of NET-G3 and NEC-G3 that displayed distinct clinical, pathologic, and molecular features, we showed a significant difference in responsiveness to platinum-based chemotherapy. NET-G3 did not respond to platinum-based regimens. We found loss of Rb immunolabeling and/or *KRAS* mutation correlated with the therapeutic response to platinum-based chemotherapy for patients with PanNEN-G3, and Rb loss correlated with response to platinum-based agents in patients with NEC-G3. Our results indicate that not only the histologic distinction but these markers are useful for the indication of platinum-based chemotherapy against PanNEN-G3.

Introduction

Since the World Health Organization (WHO) classification of 2010 incorporated a grading system, pancreatic neuroendocrine neoplasms (PanNEN) with a mitotic count of >20/10 high-power fields and/or a Ki67-labeling index (LI) of >20% are graded into grade 3 (G3; ref. 1). Pancreatic neuroendocrine carcinoma (NEC) is a rare malignant neoplasm, invariably classified as PanNEN-G3 and defined by poorly differentiated histology. Because PanNEN-G3 is rare, studies of the chemotherapeutic treatment for patients with PanNEN-G3 were limited, and current guidelines recommend platinum-based chemotherapy based on the analogy with small-cell lung carcinoma (2–4).

However, although the response rate (RR) of pulmonary NEC to platinum-based chemotherapy was high (3), the RR of extrapulmonary NEC to platinum-based chemotherapy was only 30.8% in one study (5). This discrepancy was further highlighted by other studies: the NORDIC NEC study showed that gastroenteropancreatic NEC (GEP-NEC) with a Ki67-LI of $\leq$55% responded poorly to platinum-based chemotherapy (6, 7). Recently, multiple studies suggested that PanNEN-G3 defined by the WHO 2010 classification contains another type of tumors, designated as well-differentiated neuroendocrine tumor (or NET-G3) that is characterized by well-differentiated morphology but harboring grade 3 proliferative activity (2, 8–12). NET-G3 is a rare condition, and its clinical features are not clearly known. A few small-scale studies noted the low RR of NET-G3 to platinum-based chemotherapy (13–16). The NORDIC NEC study (6) suggested that a Ki67-LI cutoff of 55% may serve as a biomarker for low response to platinum-based chemotherapy; however, it did not take morphologic classification into account, which poses a question as to whether two tumors with Ki67-LI >55% but with different histologic features equally show good response to platinum-based chemotherapy. The selection of treatment and the stratification of patients with PanNEN-G3 remain to be improved.

To address the abovementioned issues, we designed a retrospective, multicenter study of this rare but high-grade neoplasm. Seventy patients with PanNEN-G3 were analyzed in cooperation with 31 institutions in Japan. The clinicopathologic features of NET-G3 and NEC-G3, including prognosis with chemotherapy and potential predictors of therapeutic benefit, are presented.

Patients and Methods

Enrollment of patients

Thirty-one institutions in Japan participated in this study. Inclusion criteria were as follows: (i) presence of neuroendocrine features and positive labeling with at least one neuroendocrine marker (chromogranin A or synaptophysin); (ii) primary site diagnosed as the pancreas; and (iii) tumors fulfilling the WHO 2010 classification of NEN-G3 of the digestive system [mitotic count >20/10 high-power fields (HPF) and/or a Ki67-LI >20%]. The medical records at each institution were input into a standardized clinical research form, and the deidentified data (clinical information and histologic glass slides) were sent to Aichi Cancer Center Hospital (Nagoya, Japan) for central review. The collected patients were diagnosed from August 2002 to March 2015. The glass slides [hematoxylin and eosin (H&E)-stained and immunostained] were then reassessed to confirm NEN-G3. Pathologic review was performed by W. Hosoba and Y. Yatabe. Cases were excluded (i) if differential diagnoses other than neuroendocrine neoplasm were considered even after performing additional IHC analysis or (ii) if proliferative status of grade 3 was not confirmed in cases with the diagnosis of well-differentiated NET (that is, when the distinction between NET-G2 and NET-G3 was impossible). This study was conducted with the approval of the Institutional Review Boards of the 31 participating institutions.

Histologic evaluation

Having passed the central review, each case was further categorized into three subgroups [NET-G3 versus poorly differentiated NEC (NEC-G3), with NEC-G3 further grouped into small-cell NEC (SCNEC) and large-cell NEC (LCNEC)]. Specifically, NEC-G3 was characterized by tumors showing high-grade cytologic atypia, apparent pleomorphism, and

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extensive necrosis, in addition to prominent mitotic activity. NEC-G3 consisting of highly atypical cells with small- to medium-sized nuclei, finely granular chromatin, and inconspicuous nucleoli was further categorized as SCNEC. NEC-G3 with large nuclei, coarse chromatin, and well-visible nucleoli with nested proliferation was categorized as LCNEC. In contrast, tumors whose cytologic features overlapped with those of NET-G2 were also identified. The neoplastic cells displayed a low nuclear to cytoplasmic ratio and small-sized to medium-sized, ovoid nuclei, proliferating with minimal findings of pleomorphism and extensive necrosis. These tumors were designated NET-G3 and were analyzed separately from NEC-G3. The differential diagnosis of PanNEN-G3 may sometimes be difficult as reported previously (17), and in cases where multiple differential diagnoses were raised, the two pathologists discussed and reached the final diagnosis using a multiheaded microscope. Tumors were graded by calculating both the mitotic count and Ki67-LI (for measurement, see below), and if grading given by the mitotic count differed from that by Ki67-LI, the higher grade was applied (18). For fine-needle aspiration (FNA) and biopsy specimens, if areas of tumor cells did not exceed the size equivalent to 10 HPFs on microscopy, counting of the mitotic rate was not possible, and grading was carried out by Ki67-LI.

IHC and Ki67-LI

Using unstained slides having sent from the participating institutions, IHC for chromogranin A (clone SP12, rabbit, 1:200, Neomarkers), synaptophysin (clone SP11, rabbit, 1:100, Neomarkers), Ki67 (clone SP6, rabbit, 1:200; Neomarkers), and Rb (clone 3H9, mouse, 1:300; MBL) was performed. Additional immunostaining was performed for the purpose of differential diagnosis if necessary.

To minimize the interlaboratory and interobserver variability of Ki67-LI (19), measurement of Ki67-LI was centrally reviewed using Ki67 slides restained at Aichi Cancer Center Hospital. The LI was then calculated by an automated counting method, as described previously (20). Briefly, slides were digitally scanned using ScanScope XT (Aperio Technologies), and the LI was calculated using the Nuclear Image Analysis tool (Aperio Technologies). All sections were visually inspected to exclude portions with extensive desmoplasia, necrosis, and regions with bleeding. If a tumor showed different LIs among areas, the Ki67-LI was determined at the area showing the highest index. In addition, on calculating Ki67-LI on FNA/biopsy specimens, we set a criterion of 2,000 cells counted at clusters of tumor cells for the measurement of Ki67-LI; this criterion was based on the previous study for a reliable estimation of grading NEN using FNA samples (20).

Rb immunolabeling was defined as abnormal when tumor cells specifically showed loss of expression and the surrounding nonneoplastic cells retained positive nuclear staining (21).

Mutation analysis of KRAS

Mutation analysis was performed using either fresh specimens or formalin-fixed paraffin-embedded sections. Tissues were macrodissected with the aid of H&E staining. Mutation analysis of *KRAS* codon 12 was performed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) or the Cycleave PCR assay (Takara) as described previously (22, 23).

Statistical analysis

Patient response to treatment was evaluated according to RECIST, version 1.1. The disease control rate (DCR) was defined as the population of persons with complete response, partial response, or stable disease. Progression-free survival (PFS) was defined as the time from initiation of chemotherapy to confirmation of disease progression or death due to any cause, whichever came first, or the last date of follow-up. Overall survival (OS) was defined as the time from initiation of chemotherapy to death due to any cause or the last date of follow-up. Surviving patients were censored on their last follow-up date. PFS and OS were estimated using the Kaplan–Meier method and compared with the log-rank test. Uni- and multivariable Cox regression analyses were used to evaluate the impact of selected clinicopathologic factors. Differences in characteristics by groups were tested by Fisher exact test or the Mann–Whitney *U* test, as appropriate. Statistical analysis was performed using STATA, version 13.2 (STATA Corp.). Given the exploratory nature of the study, a *P* value less than 0.05 was defined as significant.

Results

The clinical and histologic information of 100 patients was gathered from the 31 participating institutions. The patient profiles are presented in Fig. 1. Thirty patients were excluded from further analysis after pathologic review. Of these, 19 patients were excluded because differential diagnoses other than neuroendocrine neoplasm could not be ruled out (Fig. 1). Two patients were excluded due to the presence of an adenocarcinoma component, and 6 patients were downgraded to NET-G2. Although 3 patients were histologically diagnosed as neuroendocrine tumors, they were excluded due to lack of effective Ki67 slides or due to crush artifact that did not allow reliable counting.

Tissue specimens were obtained through surgical resection ($n = 24$), autopsy ($n = 1$), endoscopic ultrasound-guided FNA (EUS-FNA; $n = 24$) and biopsy ($n = 21$). The site of specimens taken included the pancreas ($n = 44$; 21 by resection, 20 by EUS-FNA and 3 by biopsy), liver ($n = 21$; 3 by resection, 15 by biopsy, 2 by EUS-FNA and 1 by autopsy), and others [$n = 5$; lymph node ($n = 2$), subcutis ($n = 1$), duodenum ($n = 1$), and lung ($n = 1$); 2 by EUS-FNA and 3 by biopsy].

Histologic review of 70 patients with PanNEN-G3 was performed and patients were divided into 21 NETs-G3 (30%), 31 SCNECs (44.3%), and 18 LCNECs (25.7%).

Clinical characteristics of pancreatic NEN-G3

The clinical characteristics of the 70 patients are presented in Table 1. Eastern Cooperative Oncology Group performance status 0/1/2 was 46/19/5, respectively. Symptoms included abdominal pain (37 patients), jaundice (11 patients), weight loss (5 patients), diarrhea (3 patients), and aggravation of diabetes (1 patient).

Factors that differed significantly between NET-G3 and NEC-G3 included contrast behavior on CT ($P = 0.02$). NET-G3 cases tended to have a significantly maintained vascularity on contrast-enhanced CT. Clinically, there were no significant differences between SCNEC and LCNEC.

Pathologic features of pancreatic NEN-3

The pathologic characteristics are presented in Table 2 and Supplementary Fig. S1. The measurement of mitotic count was

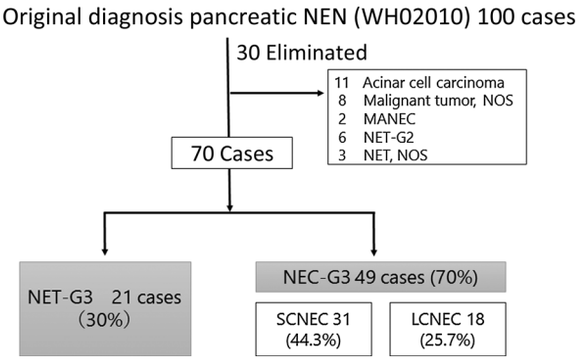

Figure 1.

Case selection. A total of 100 patients originally diagnosed as having pancreatic neuroendocrine carcinoma were included in this study. After 30 cases were eliminated, 70 cases were further divided into NET-G3 and NEC-G3 based on morphology and proliferative index. NOS, not otherwise specified; MANEC, mixed adenoneuroendocrine carcinoma.

possible in 24 resected cases. The median mitotic count of NET-G3 was 16.2/10 HPFs (2–27), and that of NEC-G3 was 44/10 HPFs (12–117). The distribution of Ki67-LI among 3 subtypes of PanNEN-G3 was examined. The measurement of Ki67-LI was assessed in 64 cases using restained Ki67 slides. Six biopsied cases were removed from this analysis (3 small-cell NECs, 2 large-cell NECs, and 1 NET-G3); four biopsied tissues allowed histologic diagnoses of SCNEC and LCNEC, but the amount of tumor cells in clusters did not reach the criterion of 2,000 cells; one biopsied tissue allowed histologic diagnosis of well-differentiated neuroendocrine tumor, and the Ki67 staining done by the collaborating hospital showed grade 3 status, but restained slides of Ki67 did not reach the 2,000-cell criterion, and one necropsy case allowed histologic diagnosis of SCNEC, but Ki67 failed to stain. The median Ki67-LI of PanNEN-G3 was 70% (15%–100%). When the Ki67-LI was compared between NET-G3 and NEC-G3, the median Ki67-LI was significantly lower in NET-G3 (28.5%) than in NEC-G3 (80.0%, $P < 0.001$). In this analysis, we included two resected cases whose mitotic count was >20/HPF but Ki67-LI was <20% (one showed 26/HPF and Ki67-LI of 15%, and the other showed 25/HPFs and Ki67-LI of 18%).

Loss of Rb expression was not observed in NET-G3 (0%), whereas NEC-G3 showed loss of expression in 54.5% of cases ($P < 0.001$). *KRAS* mutations were not detected in NET-G3, whereas NEC-G3 harbored *KRAS* mutations in 48.7% of cases ($P < 0.001$). There were no significant differences between SCNEC and LCNEC in the prevalence of abnormal Rb expression and *KRAS* mutation.

Response to treatment and OS

The treatment characteristics are presented in Table 3. Surgery was performed for 25 patients (11 NETs-G3 and 14 NECs-G3). Of 11 operated NET-G3 cases, R0/1/2 were 6/2/3, respectively. Of 14 operated NEC-G3 cases, R0/1/2 were 8/2/4, respectively. One case of NEC-G3 underwent gastrointestinal bypass surgery for palliative care. Except one bypass operation, histology was performed at the time of operation. No cases were given neoadjuvant chemotherapy. Chemotherapy was given to 58 patients (including 15 patients who underwent surgery and chemotherapy), and responsiveness could be evaluated in 55 patients. The details of the 55 patients' regimens are shown in Supplementary Table S1. Three patients were excluded because of the short duration of chemotherapy.

There were significant differences in RR and DCR between patients with NET-G3 and those with NEC-G3, with a significantly worse RR and DCR in patients with NET-G3 ($P < 0.001$). The RR of first-line platinum-based chemotherapy regimens was significantly different between NET-G3 and NEC-G3; patients with NET-G3 did not respond to first-line platinum-based chemotherapy at all (RR 0%, 0/8), whereas most of those with NEC-G3 did (61.3%, 19/31; $P < 0.001$). As for the response to chemotherapy, no significant difference between SCNEC and LCNEC was found.

A breakdown of the treatment regimens, RR, and DCR of the 16 patients with NET-G3 is presented in Supplementary Table S2. It is noteworthy that everolimus, an mTOR inhibitor effective in controlling well-differentiated NET-G1/G2, failed to suppress tumor growth in all 3 patients with NET-G3.

The median survivals were 41.8, 11.3, and 6.2 months for the NET-G3, SCNEC, and LCNEC groups, respectively, showing a significantly better prognosis for the NET-G3 ($P = 0.0023$). The HR of NET-G3 was 2.87 [95% confidence interval (CI), 1.3–6.3] for SCNEC and 3.16 (95% CI, 1.4–7.3) for LCNEC.

Correlations between the platinum-based chemotherapy response and molecular markers, including *KRAS* mutation and

Table 1. Characteristics of the 70 patients with PanNEN-G3

			NEC-G3 ($n = 49$)		*P*	*P*
	Total	**NET-G3 ($n = 21$)**	**SCNEC ($n = 31$)**	**LCNEC ($n = 18$)**	**NET-G3 vs. NEC-G3**	**SCNEC vs. LCNEC**
Age, y	64	63	64 (35–84)			
Median (range)	(30–84)	(30–81)	64 (35–76)	62 (39–84)	0.94	0.35
Sex			34:15			
Male: Female	45:25	11:10	23:8	11:7	0.24	0.35
Symptoms	81.4%	71.4%	85.7%			
Yes (%)	(57/70)	(15/21)	83.9% (26/31)	88.9% (16/18)	0.31	1.00
Tumor location	29/41	9/12	20/29			
Head/body & tail (%)	(41/59)	(43/57)	12/19 (39/61)	8/10 (44/56)	0.91	0.77
Tumor size, mm	40.5	40	42			
Median (range)	(11–150)	(20–80)	40 (11–125)	42.5 (20–150)	0.74	0.85
ENETS stage	7/10/53	4/2/15	3:8:38			
II/III/IV (%)	(10/14/76)	(19/10/71)	2/4/25 (6/13/81)	1/4/13 (6/22/72)	0.26	0.37
Vascularity on CT hyper/marginal/hypo (%)	12/9/48	7/2/12	5:7:36			
	(17/13/70)	(33/10/57)	4/1/25 (13/3/83)	1/6/11 (8/67/23)	0.02	0.58

Table 2. Pathologic characteristics of 70 patients with PanNEN-G3

			NEC-G3 ($n = 49$)		*P*	*P*
	Total	NET-G3 ($n = 21$)	SCNEC ($n = 31$)	LCNEC ($n = 18$)	NET-G3 vs. NEC-G3	SCNEC vs. LCNEC
Positive chromogranin A	92.1% (58/63)	100% (19/19)	88.6% (39/44)			
			88.5% (23/26)	88.9% (16/18)	0.31	0.20
Positive synaptophysin	94.3% (66/70)	95.2% (20/21)	93.8% (46/49)			
			96.8% (30/31)	88.9% (16/18)	1.00	0.55
Median Ki67-LI (range)	70.0% (15–100) (n=64)	28.5% (15–53)	80.0% (22%–100%)			
			85.0% (50–100)	70.0% (22–90)	<0.001	0.19
Loss of Rb expression 65 cases	36.9% (24/65)	0% (0/21)	54.5% (24/44)			
			59.2% (16/27)	47.0% (8/17)	<0.001	0.54
KRAS mutation	32.3% (20/62)	0% (0/21)	48.7% (20/41)			
			48% (12/25)	50% (8/16)	<0.001	1.00
Loss of Rb expression +*KRAS* mutation	19.6% (12/61)	0% (0/21)	30% (12/40)			
			36% (9/25)	20% (3/15)	<0.001	0.29

Rb immunolabeling, are presented in Table 4. For the 16 patients with mutated *KRAS*, the RR to platinum-based chemotherapy was significantly higher (77%) in the first-line than for patients with no *KRAS* mutation (RR = 23%; $P = 0.023$). Similarly, for the 17 patients with loss of Rb immunolabeling, the RR to platinum-based chemotherapy was significantly higher (RR = 80%) in the first-line than for patients with retained Rb immunolabeling (RR = 24%; $P = 0.006$). Furthermore, when both a *KRAS* mutation and lack of Rb immunolabeling (double aberration group; $n = 8$) were seen, the RR for platinum-based chemotherapy in the first line was 100% (8/8), whereas when no *KRAS* mutation and loss of Rb immunolabeling (double retained group; $n = 17$) was seen, the RR was 17.6% (3/17; $P < 0.001$).

The results for the predictive factors of the response to platinum-based chemotherapy in PanNEN-G3 patients are presented in Table 5. Both loss of Rb immunolabeling and *KRAS* mutations were strong predictors of the response to platinum-based chemotherapy (OR = 16.5; 95% CI, 2.69–101.33 and OR = 7.5; 95% CI, 1.49–37.65, respectively). Furthermore, when we excluded NET-G3 and analyzed 49 patients diagnosed with NEC-G3, only retained Rb immunolabeling group showed significantly worse response for platinum-based chemotherapy compared with loss of Rb ($P = 0.031$; Table 4). Loss of Rb immunolabeling was only a predictor of platinum-based chemotherapy response even in NEC-G3 (OR = 7.7; 95% CI, 1.16–51.1; $P = 0.035$; Table 5).

The factors related to OS are presented in Supplementary Table S3. On univariate analysis, a Ki67-LI of >55%, abnormal Rb expression, *KRAS* mutation, and morphologic NEC-G3 were all significant predictors of a poor prognosis; however, on multivariate analysis, only abnormal Rb expression was an independent prognostic factor.

Discussion

The current multicenter study is one of the largest studies of PanNEN-G3, analyzing 70 patients with a special focus on the correlation with responsiveness to platinum-based chemotherapy. This study allowed us to draw three conclusions: (i) NET-G3 showed distinct clinicopathologic and molecular characteristics from NEC-G3; (ii) patients with NEC-G3 responded well to platinum-based chemotherapy, whereas no remarkable response was seen in patients with NET-G3; and (iii) loss of Rb immunolabeling and *KRAS* mutation were strong predictors of response to platinum-based chemotherapy in PanNEN-G3, and loss of Rb immunolabeling was also an independent prognostic factor in PanNEN-G3, and loss of Rb immunolabeling was only a predictor of platinum-based chemotherapy response even in NEC-G3. Loss of Rb expression and *KRAS* mutation were specifically detected in NEC-G3. Our results are in agreement with others showing that Rb abnormalities were not seen in NET-G3 but were prevalent in NEC-G3 (42%–71.4%; refs. 11, 12, 21, 24). Although cases of past

Table 3. Treatment characteristics of 70 patients with PanNEN-G3

			NEC-G3 (49)		*P*	*P*
	Total	NET-G3 ($n = 21$)	SCNEC ($n = 31$)	LCNEC ($n = 18$)	NET-G3 vs. NEC-G3	SCNEC vs. LCNEC
Treatment[a] Operation/Chemotherapy/BSC	25/58/2	11/16/0	14/42/2			
			7/28/1	7/14/1	0.22	0.54
RR to chemotherapy	35.7% (20/55)[b]	0% (0/16)	51.3% (20/39)			
			57.1% (16/28)	36.4% (4/11)[b]	<0.001	0.30
DCR to chemotherapy	60.7% (34/55)[b]	37.5% (6/16)	71.8% (28/39)			
			78.5% (22/28)	54.5% (6/11)[b]	<0.001	0.23
RR to platinum-based regimen (first line)	48.7% (19/39)	0% (0/8)	61.3% (19/31)			
			68.2% (15/22)	44.4% (4/9)	<0.001	0.25
RR to platinum-based regimen (total lines)	43.1% (19/44)	0% (0/10)	55.9% (19/34)			
			60% (15/25)	44.4% (4/9)	<0.001	0.35
Median survival (months)	26.7	41.8	8.5			
			11.3	6.2	0.0023	0.036

Abbreviation: BSC, best supportive care.

[a]Of these, 15 patients underwent both surgery and chemotherapy.

[b]Three cases were not included because the response to chemotherapy could not be evaluated.

Table 4. Relationship between response to platinum-based chemotherapy and *KRAS*/Rb status in NEN-G3

***KRAS* mutation analysis for all NEN-G3 (44 cases treated with platinum-based chemotherapy)**			
	Mutated *KRAS* ($n = 16$)	**Wild-type *KRAS* ($n = 28$)**	
Platinum-based chemotherapy	**All cases were NEC-G3**	**Including 15 NEC-G3**	***P***
First-line RR	77% (10/13)	23% (6/26)	*0.023*
Total line RR	63% (10/16)	21% (6/28)	*0.006*
***KRAS* mutation analysis for only NEC-G3 (31 cases)**			
Platinum-based chemotherapy	**Mutated *KRAS* ($n = 16$)**	**Wild-type *KRAS* ($n = 15$)**	***P***
First-line RR	77% (10/13)	40% (6/15)	*0.055*
Total line RR	63% (10/16)	40% (6/15)	*0.186*
Rb immunostaining for all NEN-G3 (41 cases treated with platinum-based chemotherapy)			
	Loss of Rb ($n = 17$)	**Retained Rb ($n = 24$)**	
Platinum-based chemotherapy	**All cases were NEC-G3**	**Including 14 NEC-G3**	***P***
First-line RR	80% (12/15)	24% (5/21)	*0.006*
Total line RR	71% (12/17)	21% (5/24)	*0.003*
Rb immunostaining for only NEC-G3 (31 cases)			
Platinum-based chemotherapy	**Loss of Rb ($n = 17$)**	**Retained Rb ($n = 14$)**	***P***
First-line RR	80% (12/15)	38.4% (5/13)	*0.031*
Total line RR	71% (12/17)	35.7% (5/14)	*0.057*
***KRAS* mutation & Rb immunostaining for all NEN-G3 (40 cases with platinum-based chemotherapy)**			
	Mutated KRAS & loss of Rb ($n = 10$)	**Wild-type *KRAS* & retained Rb ($n = 19$)**	
Platinum-based chemotherapy	**All cases were NEC-G3**	**Including 9 NEC-G3**	***P***
First-line RR	100% (8/8)	17.6% (3/17)	*<0.001*
Total line RR	80% (8/10)	15.7% (3/19)	*0.001*
***KRAS* mutation & Rb immunostaining for only NEC-G3 (19 cases)**			
Platinum-based chemotherapy	**Mutated KRAS & loss of Rb ($n = 10$)**	**Wild-type *KRAS* & retained Rb ($n = 9$)**	***P***
First-line RR	100% (8/8)	33.3% (3/9)	*0.007*
Total line RR	80% (8/10)	33.3% (3/9)	0.055

studies were limited, they also revealed *KRAS* mutations in NEC-G3, while PanNETs-G1/G2 with *KRAS* mutations were rarely reported (21, 22, 25). Analyses of Rb immunolabeling and *KRAS* mutation can be useful adjuncts to distinguish NET-G3 from NEC-G3.

The current study suggests that Rb loss and *KRAS* mutation are potential predictors of response to treatment for PanNEN-G3. Platinum-based agents are toxic to patients, often causing severe side effects that resulted in lowering performance status. Our results show that NEC-G3 with Rb loss would be an indication for platinum-based chemotherapy. On the contrary, NEC-G3 with retained Rb was found to show significantly worse response for platinum-based chemotherapy. However, there is no evidence what chemotherapy should we use for these category (24, 26, 27). Further studies are needed (28).

Although patients with NET-G3 did not respond to platinum-based chemotherapy in the current study, NET-G3 patients failed to respond to non–platinum-based chemotherapy in all 8 cases. These cases included 3 patients treated with everolimus. Although antitumor effects of everolimus were not tested in the previous randomized clinical trials that focused on pancreatic well-differentiated NET-G1/G2, it was reasonably expected that everolimus may suppress the growth of NET-G3, considering the fact that NET-G3 shows a close resemblance to NET-G2 in clinicopathologic and molecular features (29). There were small-sized studies that reported NET-G3 patients who responded to everolimus (30–32). Alkylating agents were also reported to be effective against NET-G3 in one study (14). We expect further studies will explore effective chemotherapeutic regimens in controlling advanced NET-G3 (27, 28).

Table 5. Predictive factors for first-line platinum-based regimen

All PanNEN-G3		
	OR (95% CI)	***P***
Loss of Rb immunolabeling	16.5 (2.69–101.33)	0.002
KRAS mutation	7.5 (1.49–37.65)	0.014
Ki67-LI > 55%	10.7 (1.84–62.5)	0.008
Loss of Rb or *KRAS* mutation	9.28 (1.98–43.4)	0.005
Only NEC-G3		
	OR (95% CI)	***P***
Loss of Rb immunolabeling	7.7 (1.16–51.1)	0.035
KRAS mutation	3.49 (0.64–19.2)	0.149
Ki67-LI > 55%	2.14 (0.25–18.5)	0.488
Loss of Rb or *KRAS* mutation	3.57 (0.66–19.3)	0.140

The clinicopathologic difference between LCNEC and SCNEC was examined. LCNEC and SCNEC shared similar clinical and molecular findings and responsiveness to chemotherapy, except that SCNEC showed better prognosis than LCNEC ($P = 0.036$). Another study of 44 pancreatic NECs by Basturk and colleagues (17) found that the difference in survival between these two groups was not significant. Considering limitations including sample size and potential selection bias, further studies on the prognostic difference would be needed.

We found that half of the SCNECs had *KRAS* mutations. Interestingly, this is in contrast to small-cell carcinoma of the lung in which *KRAS* mutations are rarely found (33). In addition, although the prevalence of inactivated Rb is high (90%) in small-cell lung carcinoma, loss of expression of Rb was seen in 60% of SCNECs in this study (34). Our results suggest that abrogation of the Rb signaling pathway plays an important role in forming small-cell carcinoma of the pancreas as well and, at the same time, that another mechanism of tumorigenesis unique to pancreatic SCNEC is present. The finding of frequent *KRAS* mutations also allows us to hypothesize that pancreatic SCNEC is of ductal origin, or that the

relevance of mutated *KRAS* is different from that of ductal neoplasms.

The limitations of this study include a potential selection bias of patients due to the retrospective design and it being a multicenter study. Thus, it is not possible to accurately estimate the prevalence of NET-G3 in PanNEN-G3 from this study. Furthermore, because many NENs-G3 were unresectable at the time of diagnosis, 64.2% of the tissue specimens for diagnosis were biopsy and EUS-FNA specimens, which may cause concern about the evaluation of Ki67-LI distribution among the 3 subtypes. We believe the possibility of misgrading was likely very low (particularly misgrading of G2-NET to grade 3) because of the strict standard we set for Ki67 counting.

In conclusion, NET-G3 and NEC-G3 showed distinct clinical, imaging, pathologic, and molecular features and, most importantly, different responsiveness to platinum-based chemotherapy. Loss of Rb immunolabeling and *KRAS* mutation are promising molecular markers of the therapeutic response to platinum-based chemotherapy for PanNEN-G3, and Rb for NEC-G3.

Disclosure of Potential Conflicts of Interest

M. Ueno reports receiving other commercial research support from Eizai, Merck, MSD, Ono, Shire, and Taiho, speakers bureau honoraria from AstraZeneca, Ono, and Taiho, and is a consultant/advisory board member for Eizai. M. Ikeda reports receiving speakers bureau honoraria from Novartis Pharma K.K. S. Nakamori reports receiving commercial research grants from Eizai. N. Mizuno reports receiving commercial research grants from AstraZeneca, Eisai, Merck Serono, MSD, NanoCarrier, Taiho Pharmaceutical, and Zeria Pharmaceutical, and speakers bureau honoraria from Kyowa Hakko Kirin, Novartis, Pfizer, Taiho Pharmaceutical, and Yakult Honsha. No potential conflicts of interest were disclosed by the other authors.

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