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Original Article

Long-term outcomes of World Health Organization G3 pancreatic neuroendocrine neoplasms: a retrospective study in combination of morphological and proliferative analysis

Yang Chen, Min Yang, Weiguo Wang, Mingquan Huang, Li Wang, Bole Tian

Department of Hepatobiliopancreatic Surgery, West China Hospital of Sichuan University, China

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Abstract: Background: The prognostic assessment of pancreatic neuroendocrine neoplasms (p-NENs) has been significantly improved since systematical classification of this disease into three grades were recommended by the 2010 World Health Organization (WHO) guidelines in which G3 p-NEN is equal to pancreatic neuroendocrine carcinoma (p-NEC) with a poor prognosis. Limited investigations recently uncovered a subgroup of G3 p-NENs morphologically well-differentiated with better survival outcomes compared with the traditional poorly-differentiated p-NENs, prompting us to assess the clinical and pathologic characteristics and prognosis of patients with G3 p-NENs. Methods: The databases for patients pathologically diagnosed as G3 p-NENs of the author's institution from January 2003 to December 2015 were identified and analyzed retrospectively, as well as morphological differentiation according to the WHO 2000 classification. Results: Overall, 76 patients with pathologically confirmed G3 p-NENs were incorporated in our study, in which 21 (27.6%) and 55 (72.4%) cases showed well differentiated or poorly differentiated morphological features, defined as G3 p-NETs and G3 p-NECs respectively. Patients with G3 p-NETs were significantly more likely to have a less-progressive (P=0.0038) and lymph-negative (P=0.004) tumor against G3 p-NECs. Median Ki-67 positive index in G3 p-NECs (42%, range: 22-75) was significantly higher than in G3 p-NETs (12%, range: 4-26; P<0.001). Those with G3 p-NETs had significantly longer overall survival (OS) than ones with G3 p-NECs (P<0.001). Morphological differentiation (HR, 0.311; P<0.001), tumor stage (HR, 0.362; P=0.009) and surgical margin status (HR, 0.534; P=0.013) were all independent predictors for OS of this disease. Conclusion: Our studies provide credible evidences on heterogeneity of G3 p-NENs including poorly-differentiated ones with aggressive nature and well-differentiated with better survival outcomes. The current WHO classification of G3 p-NENs seems controversial and further modification is warranted, thus further prospective clinical trials are necessary to provide more convincing grading classifications for clinicians.

Keywords: Pancreatic neoplasm, neuroendocrine tumor, Ki-67 index, WHO classification, overall survival, surgical margin status, morphological differentiation

Introduction

Pancreatic neuroendocrine neoplasms (p-NENs) are relatively rare group of tumors with pathological heterogeneity and potential malignancy, which may derive not only from mature pancreatic endocrine cells but also from pluripotent stem cells of the pancreas [1-3]. Due to the significant improvement of awareness for this disease and wide applications of imaging technologies for physical examination, p-NENs, though still accounting for <3% of all pancreatic neoplasms, have presented an obviously increasing incidence in the past 2 decades [4, 5]. The prognosis of p-NENs varies considerably as poorly-differentiated lesions are almost equal to typically clinical-aggressive malignancies with pretty poor outcomes, while well-differentiated ones may present a relatively good long-term survival [6].

In view of the rarity and heterogeneous behavior of p-NENs, the stratification of patients with p-NENs for prognostic analysis has been consistently challenging. Morphological differentiation has been previously validated in correla-

tion with the prognosis of p-NENs [7-9]. Further studies have also verified the applications of proliferation activity to stratify prognostic subgroups of these tumors, which was mainly assessed by mitotic rate and Ki-67 positive index [10, 11]. Systematic grading, based on proliferative activity along with morphological differentiation, was put forward in 2006 by the European Neuroendocrine Tumor Society (ENETS) [12] and then incorporated into the 2010 World Health Organization (WHO) classification of digestive neuroendocrine neoplasms [13]. This WHO grading system has divided p-NENs into three classifications which mainly refers to proliferative activity and Ki-67 positive index: G1) pancreatic neuroendocrine tumor (p-NET) with <2 mitotic figures/10 high-power fields (HPF) and a Ki-67 index of <3%; G2) p-NET with 2 to 20 mitotic figures/10HPF or a Ki-67 index of 3% to 20%; G3) pancreatic neuroendocrine carcinomas (p-NECs) with >20 mitoses/10 HPF or Ki-67 index >20%.

According to the WHO 2010 classification, p-NENs in G1 and G2 are commonly regarded as well-differentiated tumors whereas G3 p-NENs are considered equally to poorly-differentiated carcinomas. However, significantly heterogenous responses to platinum-based chemotherapy within the G3 subgroup were reported recently [14]. Moreover, other publications divaricated that some well-differentiated p-NENs could manifest highly proliferative activities evaluated by mitotic rate or, more usually, Ki-67 positive index and might be accordingly classified into G3 category as well [15-17]. Thus, the current grading system for G3 p-NENs has been raising increasing controversies due to its lack of the combination of morphological and proliferative analysis. Based on the data from our single center, our research was designed to analyze and compare the clinical and pathologic characteristics, surgical outcome and prognosis of patients with both well-differentiated and poorly-differentiated G3 p-NENs defined by the WHO 2010 criteria.

Method

Clinical information

The database for patients who were pathologically diagnosed as G3 p-NENs in the author's institution from January 2003 to December 2015 was identified and analyzed retrospectively. Available medical records were reviewed to obtain clinical data, including demographic information, clinical presentation associated with preoperative workup, imaging modalities including conventional high-resolution imaging technique along with fludeoxyglucose positron emission tomography (18-FDG PET), pathology data and further laboratory examination. Tumor staging was carried out following the seventh edition of American Joint Cancer Committee (AJCC) cancer staging manual [18]. Perioperative complications and their grading systems were classified according to the International Study Group for Pancreatic Surgery (ISGPS) definition [19-21]. The association of platinum-based regimens to etoposide was considered as the first-line neoadjuvant and adjuvant chemotherapy [22]. Follow-up was mainly conducted by out-patient clinic or telephone every 3 to 6 months with conventional clinical examination, laboratory analyses and imaging inspection.

Pathological assessment

Hematoxylin and eosin stained slides (average 5 per case) of operative or biopsy specimens from patients with G3 p-NENs were evaluated independently by two pathologists with expertise. Further discussion would be implemented by both pathologists in case of discrepancy. The mitotic rate of each case was determined by counting mitotic figures in 50 HPFs (1 HPF=0.25 mm^2) and averaged to 10 counts as the final report. The Ki-67 positive index was expressed as the percentage of tumor cells with nuclear staining, based on counting >2000 cells in the regions with the most intensely labeling regions (hot spots). Strong intensity of p53 immunoreactivity in >25% tumor cells and loss of Rb protein expression were regarded as abnormal. Furthermore, pathological files also included tumor size, stage by AJCC, resection margin status, functional analysis and other details.

The morphological classification in our study was based on the WHO 2000 criteria [23], while the proliferative analysis was referring to the WHO 2010 grading system [13]. The characteristics of well-differentiated p-NENs were marked by typical neuroendocrine architectural tissues with organoid features and tumor cells with low nucleocytoplasmic ratio, abundant

Table 1. Clinical and pathological features of the entire cohort with G3 p-NENs

Variable	Total	G3 p-NETs	G3 p-NECs	*P* value
Age at diagnosis, year				
Median, (range)	56 (26-73)	54 (32-73)	57 (26-71)	0.517
Gender, N (%)				
Female	43 (56.6)	14 (66.7)	29 (52.7)	
Location, N (%)				0.434
Head/uncinate	27 (35.5)	6 (28.6)	21 (38.2)	
Body/tail	49 (64.5)	15 (71.4)	34 (61.8)	
Tissue, N (%)				0.054
Biopsy	19 (25.0)	2 (9.5)	17 (30.9)	
Surgery	57 (75.0)	19 (90.5)	38 (69.1)	
Tumor diameter, cm				
Median (range)	4.9 (1.6-12.7)	4.2 (2-7.1)	5.3 (1.6-12.7)	0.251
Stage by AJCC, N (%)				0.038
I/II	31 (40.8)	13 (61.9)	18 (32.7)	
III	19 (25.0%)	5 (23.8%)	14 (25.5)	
IV	26 (34.2)	3 (14.3)	23 (41.8)	
Functional status, (N)				
Functional	24	10	14	0.063
Lymph Node Status				0.004
N0	21	12	9	
N1	36	7	29	
Resection margin, (N)				0.601
R0	37	14	23	
RI	9	2	7	
R2	11	3	8	
Ki-67, (%)				
Median(range)	51 (20-86)	24 (20-56)	55 (23-86)	<0.001
Mitotic rate, (per 10HPS)				
Median (range)	22 (4-75)	12 (4-26)	42 (22-75)	<0.001
p53		0/8	5/12	-
Rb mutation		0/3	3/5	-
Overall Survival, months				
Median (95% CI)	22.4 (20.6-24.2)	44.7 (31.5-57.9)	18.8 (16.2-21.4)	<0.001

G3 = grade 3 by the 2010 WHO classification, p-NENs = pancreatic neuroendocrine neoplasms, p-NETs = well-differentiated pancreatic neuroendocrine tumors, p-NECs = poorly-differentiated pancreatic neuroendocrine carcinomas, HPS = high-power fields.

eosinophilic or amphophilic cytoplasm, and ovoid nuclei with salt and pepper chromatin containing well-defined nucleoli. Poorly-differentiated ones, however, were featured on nodular or solid architecture lack of organoid traits, usually with high nucleocytoplasm ratio and multifocal or extensive tumor necrosis, including small cell and large cell subtypes. As we mentioned before, the G3 p-NENs could be morphologically either well-differentiated or poorly-differentiated. For convenience in the present study, we directly defined well-differentiated p-NENs as G3 p-NETs, while poorly-differentiated ones as G3 p-NECs.

Statistical analysis

Continuous variables were reported as median value and range whereas categorical variables were presented as numbers and percentages. Normally distributed continuous variables were compared using a 2-sample Student *t* test, and

Table 2. Characteristics of imaging and treatment according to the morphological differentiation

Variable	Total	G3 p-NET	G3p-NEC	*P* value
Preoperative imaging, N				
HRCT positive	38/53	11/14	27/39	0.506
MRI positive	27/38	9/13	18/25	0.858
18-FDG PET/CT positive	14/22	5/9	9/13	0.512
Operative data, N	57	19	38	
Operating methods				0.711
Pancreatoduodenectomy	18	7	11	
Distal pancreatectomy with splenectomy	25	6	19	
Local resection	14	6	8	
Duration of stay, day				
Median (range)	13.8 (6-35)	12.5 (6-28)	14.1 (8-35)	0.283
Perioperative complications				
POPF, N (%)				
Grade ≥B	7 (12.3)	2 (10.5)	5 (13.2)	0.775
DGE, N (%)	10 (17.5)	3 (15.8)	7 (18.4)	0.805
PPH, N (%)	4 (7.0)	1 (5.3)	3 (7.9)	0.714
Mortality	1	0	1	-
Reoperation	3	1	2	-
Lymph node count, N				
Median (range)	12 (3-21)	11 (5-16)	12 (3-21)	0.659
Chemotherapy, N (%)	35 (46.1)	8 (38.1)	27 (49.1)	0.553

POPF = postoperative pancreatic fistula, DGE = delayed gastric emptying, PPH = postpancreatectomy hemorrhage, CDC = Clavien-Dindo classification.

the Mann-Whitney U test was used for abnormally distributed variables. As for categorical variables, the distributions were compared by Chi-square test or the Fisher exact test, as appropriate. Survival analyses were plotted using the Kaplan-Meier method and compared by the log-rank test. Overall survival (OS) was calculated from the time of initial diagnosis. Univariate and multivariate Cox regression analyses were performed to estimate significant predictors of OS. Relative risks were expressed as hazard ratios (HRs) with 95% confidential intervals (CIs). A *P* value of <0.05 was considered statistically significant. The SPSS statistical software version 22.0 package (SPSS, Inc.) was used for the data analyses.

Results

Clinical and perioperative features

Overall, 76 patients with pathologically confirmed G3 p-NENs were incorporated in our study. None of the patients was typically inherited. The demographic and clinical characteristics of the entire cohort were represented in **Table 1**. The median age at diagnosis of the patients was 56 years (range 26 to 73 years), with a female to male ratio of 1.30. Twenty-seven (35.5%) patients had a tumor in the head or uncinate process of pancreas, while the remaining 49 ones (64.5%) in the body or tail. For morphological features as we defined, there were in total 21 G3 p-NETs (27.6%) and 55 G3 p-NECs (72.4%), respectively.

Conventional imaging technologies including high-resolution computed tomography (HRCT) and magnetic resonance imaging (MRI) were taken as the crucial step on diagnostic process. Sensitivity of HRCT and MRI in the patients were 71.7% and 71.1% respectively (**Table 2**). Further nuclear medicine imaging by 18-FDG PET did not identify any significant difference between the two groups. After preoperative evaluation, 63 patients received surgery while the other 13 were certified with unresectable and/or metastatic tumors supported by percutaneous fine-needle biopsy. Among those undergoing operative exploration, 57 patients accepted pancreatic resection, including 37

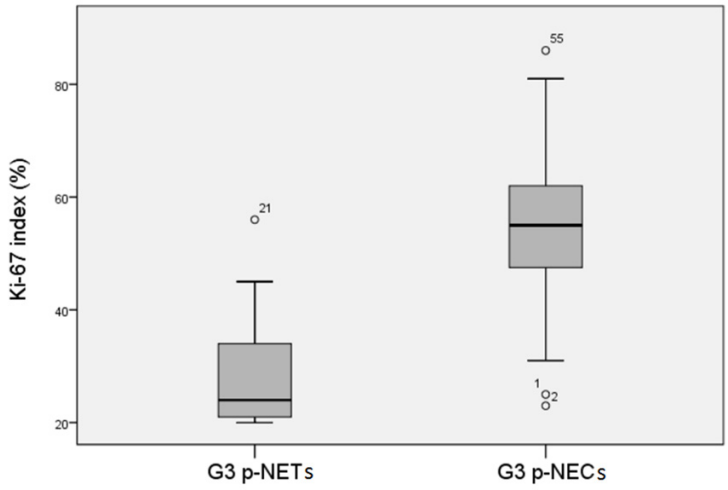

Figure 1. Distribution of Ki67 index in G3 p-NENs with morphological differentiation.

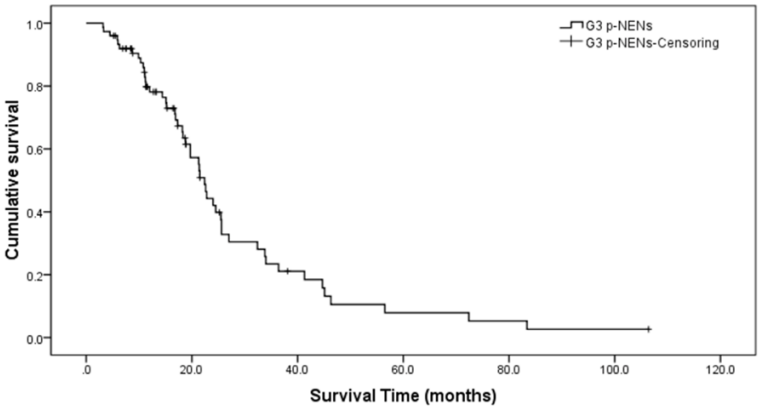

Figure 2. Survival curve of the whole patients with G3 p-NENs incorporated.

with curative resection (R0) whereas the remaining 20 underwent palliative resection (R1/R2) on account that the tumors finally turned out to be locally advanced unresectable; 6 with unresectable disease or widespread metastasis received fine-needle biopsy or aspiration of the primary pancreatic neoplasm and underwent hepaticojejunostomy or gastrojejunostomy for the obstruction of duodenum and biliary duct. As for the surgical procedures, eighteen patients had pancreatoduodenectomy, 25 had distal pancreatectomy with splenectomy, and the remaining 14 had local resection (P=0.711). Median duration of patients in hospital was 13.8 days (range 6-35, P=0.283). Perioperative complications didn't exhibit a significant difference on aspect of postoperative pancreatic fistula (POPF), delayed gastric emptying (DGE) and post-pancreatectomy hemorrhage (PPH) between G3 p-NETs and G3 p-NEC, though one was dead for acute and unexplained postoperative bleeding in PD group. Median number of lymph node count for the operative specimens was 12 (range 3-21, P=0.659).

Thirty-five patients were treated by platinum-based chemotherapy, including 8 with G3 p-NETs (6 undergoing palliative resection and 2 without surgery) and 27 with G3 p-NECs (14 with palliative resection, 13 without surgery and the other 2 receiving curative resection) (P=0.553).

Pathological features

Median tumor diameter for the patient involved was 4.9 cm (range 1.6-12.7, P=0.251). Patients with G3 p-NETs were significantly more likely to have a lessprogressive (Stage by AJCC, P=0.038) and lymph-negative (Lymph Node Status, P=0.004) tumor against G3 p-NECs (**Table 1**). The median Ki-67 index of the whole cases was 51% (range 20%-86%). The index of G3 p-NECs (55%, range: 23%-86%) was significantly higher than in G3 p-NETs (24%, range: 20%-56%; P<0.001) according to the distribution in relation to differentiation displayed in **Figure 1**. Median mitotic rate tended to be higher in G3 p-NECs (42, range: 22-75) than in G3 p-NETs (12, range: 4-26; P<0.001). Further immunohistochemical analysis indicated immunoreactivity of p53 and loss of Rb protein expression were both negative in G3 p-NETs but positive in G3 p-NECs (5/12, 3/5; respectively).

Survival outcomes

Clinical follow-up information was available for all 75 patients but 1 who died of surgical complications. The median follow-up of the studied

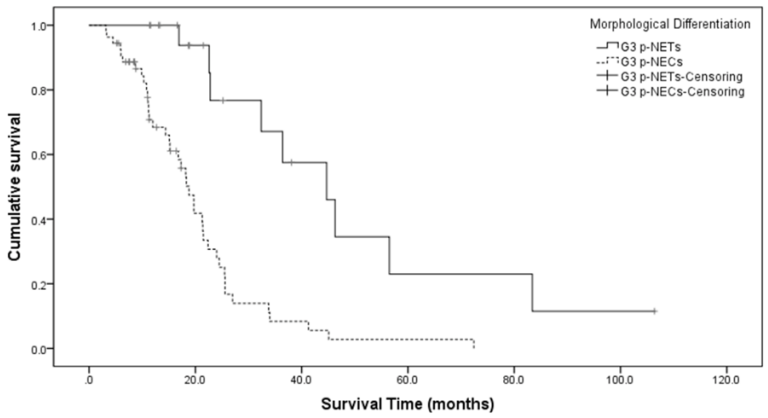

Figure 3. Survival curves of G3 p-NENs according to morphological differentiation.

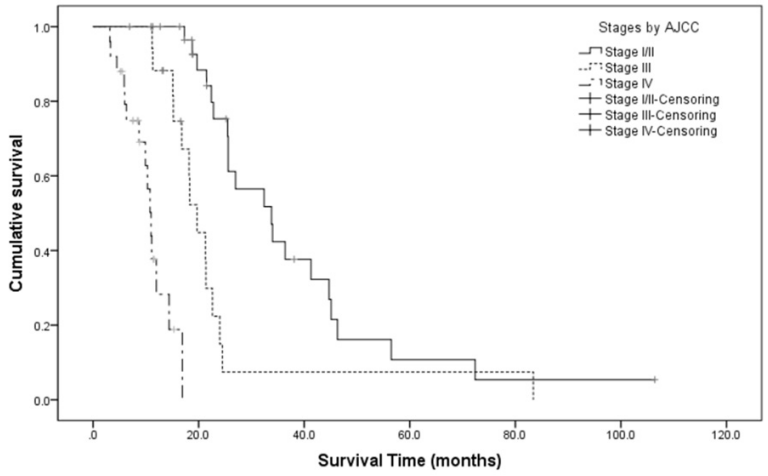

Figure 4. Survival curves of different stages by AJCC.

population was 16 (range: 3-106) months. Forty-nine patients were followed up to the time of their death: 43 died as a result of the progression of tumor and remaining six due to other causes. Median survival time for the whole patients involved was 22.4 months (95% CI, 20.6-24.2; **Figure 2**). Those with G3 p-NETs had significantly longer OS (median: 44.7, 95% CI: 31.5-57.9) than ones with G3 p-NECs (median: 18.8, 95% CI: 16.2-21.4; P<0.001; **Figure 3**). Earlier stage (P<0.001; **Figure 4**) and radical resection (P<0.001; **Figure 5**) were related to a better prognosis.

The univariate and multivariate analysis of patients with G3 p-NENs by Cox regression model indicated that morphological differentiation (HR, 0.311; P<0.001), tumor stage (HR, 0.362; P=0.009) and surgical margin status (HR, 0.534; P=0.013) were all independent predictors for OS of this disease. Patients with tumor ≤4.9 cm, functional and ki-67 index ≤51% were associated with a longer life expectancy only supported by univariate analysis, whereas multivariate Cox regression didn't show any significant difference (**Table 3**).

Discussion

The classification of p-NENs is commonly summarized as three gradings by the 2010 WHO consensus according to their mitotic rate and Ki-67 positive index, in which G3 neoplasms are generally regarded as poorly-differentiated carcinomas. Recent publications in regard to p-NENs, however, announced a significant heterogeneity of G3 neoplasms, which could originally present a high proliferative activity (i.e. Ki-67 positive index) but be morphologically well-differentiated with a better OS [24]. Based upon analyzing the clinical, operative and pathological features of G3 p-NETs and G3 p-NECs with the largest population incorporated so far, our present study has further proofed that these neoplasms were still heterogeneous which was not only reflected by the latest WHO classification.

The epidemiological distribution of subgroups of G3 p-NENs was hard to be described due to the lack of relevant studies. Recently, a prospective and epidemiological research put forward that G3 p-NETs were possibly more common than G3 p-NECs [14]. However, their study mainly expounded the outcomes of various treatments for G3 p-NENs, without any detailed discussion about their pathological characteristics, especially the morphological differentiation. In our series, contrast to the former epidemiological results, the majority of G3 p-NENs was p-NECs (72.4% vs 27.6%), implying a promi-

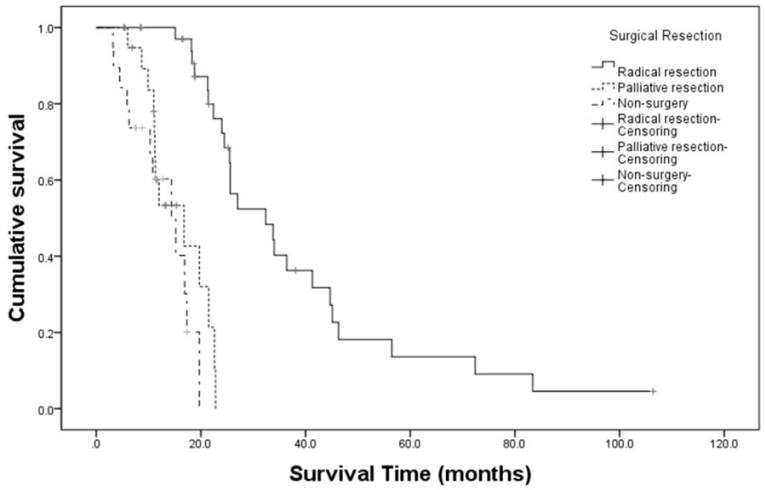

Figure 5. Survival curves of G3 p-NENs with different resection.

nently inherent consistency between proliferative activity and morphological differentiation on account that the great mass of G3 p-NENs classified on the basis of proliferation are truly malignant characterized by poorly-differentiated morphology, and the existence of G3 p-NETs, of course, couldn't be underestimated.

Despite G3 p-NETs and G3 p-NECs were both performed as aggressive diseases with neuroendocrine phenotype, G3 p-NECs were more close to the definition of a conventional carcinoma [25, 26]. A recent review reported that G3 p-NETs showed an intermediate prognosis between G2 p-NETs and G3 p-NECs [27]. In our research, lymph node metastases of the G3 p-NECs were maintained at a high level and the distant metastasis rate was up to 41.8% at the time of initial diagnosis. For the survival analysis, G3 p-NETs showed a significantly better prognosis against G3 p-NECs. In view of the limitations of our research that the database was based on pathological diagnosis and that we might lose those patients without pathologically confirmed but usually more aggressive tumors, we have reasonable grounds to believe in a more malignant nature of G3 p-NECs. All those above evidence suggested we should distinguish these two subgroups G3 p-NENs with distinct prognoses.

Immunohistochemistry plays a crucial role in the classification of p-NENs and the Ki-67 positive index has become a fundamental measurement in their management. However, mitotic rate and Ki-67 positive index might be discordant, and counting by eye observation or digital image analysis might tend to overestimate the Ki-67 index [28, 29]. In our study, G3 p-NETs had a consistently low-level mitotic rate when Ki-67 index over 20%. But for G3 p-NECs, mitotic rate and Ki-67 index both maintained rather high levels. Thus the two indexes were inharmonious to some extent. Furthermore, multivariate Cox regression analysis of OS in our study underlined the morphological differentiation as an independent predictor for the survivals of G3 pNENs instead of Ki-67 index. Genetically sporadic differences emerged recently between G3 p-NETs and G3 p-NECs, in which increasing detection measurements at molecular level were conducive to identify these two subgroups, especially when G3 p-NETs were morphologically indistinguishable from G3 p-NECs [30-32]. Abnormal immunohistochemical markers, such as p53 and the Rb pathways, were detected only in PD-pNECs according to our research. The following issues, therefore, could be further reconsidered: 1) emphasis on morphological differentiation in addition to proliferative activities; 2) modification of the Ki-67 cutoff index; and 3) integration of molecular features to better distinguish the two subtypes.

Prognosis of patients with p-NENs varies based on stage of disease at diagnosis. A review of high-grade gastrointestinal NECs incorporating 2546 patients from the SEER data indicated a up to 57% distant-metastasis rate; the median survival time for localized, locally advanced and distant disease was 38 months, 16 months and 5 months respectively [27]. Tumor stage of G3 p-NENs was also manifested as an independent predictor according to the univariate and multivariate Cox regression analyses in our research. Surgical resection is still recommended as the essential management of localized p-NENs by the ENETS and U.S. guidelines [33-35]. Curative resection of G3 p-NENs was associated with a significant prolongation of survival time against palliative resection or

Table 3. Univariate and multivariate analysis of predictors for G3 p-NENs by cox regression

Parameter	MST, mo	*P* value of Univariate	*P* value of Multivariate	Hazard Risk	95% CI
Age, year					
≤56	24.1				
>56	19.7	0.137			
Gender					
Female	18.3				
Male	25.6	0.081			
Location					
Head/Uncinate	19.5				
Body/Tail	24.8	0.372			
Size, cm					
≤4.9	27.0				
>4.9	19.7	0.005	0.279		
Function					
Yes	33.8				
No	19.7	0.002	0.489		
Morphology					
G3 p-NETs	44.7				
G3 p-NECs	18.8	<0.001	<0.001	0.311	0.153-0.606
Stage by AJCC					
I and II	33.8				
III and IV	15.2	<0.001	0.009	0.362	0.169-0.776
Radical resection					
Yes	32.4				
No	15.2	<0.001	0.013	0.534	0.325-0.876
Ki67 index (%)					
≤51	27.0				
>51	16.8	<0.001	0.347		
Chemotherapy					
Yes	23.5				
No	17.2	0.117			

MST = median survival time, CI = confidence interval, AJCC = American Joint Committee on Cancer, p-NENs = pancreatic neuroendocrine neoplasms, p-NETs = well-differentiated pancreatic neuroendocrine tumors, p-NECs = poorly-differentiated pancreatic neuroendocrine carcinomas.

non-surgery management in our series, and operative exploration for potentially curative resection should be considered precedently for this reason.

In consideration of the absence of randomized controlled trials and prospective studies about G3 p-NENs, retrospective analyses might still provide new insights for the controversial but rare disease, which was the main purpose of our studies. We had to acknowledge, nevertheless, the limitations of our research were consistent: 1) probable bias from retrospective collection of data, such as the absence of advanced G3 p-NENs without pathologically confirmation; 2) the lack of comprehensive data analyzing on molecular level of G3 p-NENs. Further in-depth or prospective study of the new-emerging subgroup is still necessary.

In conclusion, our study provided credible evidence on heterogeneity of G3 p-NENs, which included poorly-differentiated tumors with aggressive nature and well-differentiated ones with better survival outcomes. Both morphological differentiation and proliferative activity should be taken into considerations on account that identifications of G3 p-NENs are the cornerstone to better propel their diagnostic and therapeutic process. The current WHO classification for G3 p-NENs seems controversial and further modification is warranted. Further prospective clinical trials are necessary to provide more convincing grading classifications and better prognostic and predictive tools for clinicians.

Disclosure of conflict of interest

None.

Authors' contribution

CY proposed the study. CY and YM performed research and wrote the first draft. CY and WWG collected and analyzed the data. All authors contributed to the design and interpretation of the study and to further drafts. CY is the guarantor.

Address correspondence to: Bole Tian, Department of Hepatobiliopancreatic Surgery, West China Hospital of Sichuan University, Guoxue Road 37, Wuhou District, Chengdu 610041, Sichuan Province, China. E-mail: tianbole@qq.com

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