Progesterone Receptor and PTEN Expression Predict Survival in Patients With Low- and Intermediate-Grade Pancreatic Neuroendocrine Tumors

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• ***Context.***—**The PI3K-AKT-mTOR (phosphatidylinositol 3-kinase–AKT–mammalian target of rapamycin) pathway plays a crucial role in a subset of advanced pancreatic neuroendocrine tumors (PanNETs). In breast and endometrial carcinoma, activation of this pathway inhibits progesterone receptor (PR) expression.**

Objective.—**To determine whether combined low expression of PR and phosphatase and tensin homologue (PTEN), a negative regulator of the PI3K-AKT-mTOR pathway, is a prognostic factor.**

Design.—**A total of 160 resected PanNETs (89 low grade and 71 intermediate grade) were analyzed for PR and PTEN immunohistochemical positivity and staining was correlated with metastasis-free survival (MFS) and overall survival (OS). Progesterone receptor staining was scored as positive by using 1% or greater as cutoff. Weak/faint staining in greater than 90% of tumor cells was considered low PTEN positivity.**

Results.—**Most PanNETs (110 cases, 69%) were both PR and PTEN positive, 45 (28%) were either PR or PTEN positive, and only 5 (3%) had a PR-negative and PTEN-low profile. Combined PR-PTEN positivity was significantly associated with MFS in patients with stage I and II disease ($P < .001$) and OS in all patients ($P < .001$) and remained a significant predictor of survival after adjusting for other factors. Patients with PR-negative–PTEN-low PanNETs had the shortest median MFS and OS, compared to those with tumors that were either PR or PTEN positive and with tumors positive for both PR and PTEN ($P \leq .001$).**

Conclusion.—**Combined immunohistochemical assessment of PR and PTEN may help identify a small subset of PanNETs with more aggressive behavior and may aid in risk stratification.**

(*Arch Pathol Lab Med.* 2014;138:1027–1036; doi: 10.5858/arpa.2013-0195-OA)

Pancreatic neuroendocrine tumors (PanNETs), as defined by the 2010 *World Health Organization (WHO) Classification of Tumours of the Digestive System*,[1] are rare, morphologically well-differentiated neoplasms with malignant potential. The clinical behavior for PanNETs is highly variable and unpredictable. Up to 15% of patients with PanNETs present with distant metastasis,[2] and although the clinical course for these patients is protracted, they have a dismal 5-year survival rate of 20%.

Recently, grading and staging systems for PanNETs have been established to identify patients with increased risk of poor outcome and to guide clinical management, as studies have shown that the WHO grade and American Joint Committee on Cancer (AJCC) stage are the most important prognostic factors in PanNETs.[3–7] PanNETs are staged on the basis of the pancreatic cancer staging system recommended in the 2010 *AJCC Cancer Staging Manual*, 7th edition.[8] Grading of PanNETs, defined by the WHO, is based solely on the proliferative index by mitotic count or Ki-67 immunohistochemistry and separates PanNETs into low grade (G1, <2 mitoses per 10 high-power fields [HPFs] or Ki-67 labeling index <3%) and intermediate grade (G2, 2–20 mitoses per 10 HPFs or Ki-67 labeling index of 3%–20%).[1] However, the subtle difference in proliferative index between G1 and G2, compounded by intratumoral heterogeneity,[9] makes grading quite challenging, especially in biopsy specimens. Additional studies are needed to further enhance our ability to predict outcome.

Recent studies have shown that activation of the PI3K-AKT-mTOR (phosphatidylinositol 3-kinase–AKT–mammalian target of rapamycin) pathway plays an important role in a subset of PanNETs.[10–12] Patients with advanced PanNETs (ie, unresectable and metastatic) have shown improved progression-free survival with treatment with everolimus, an mTOR inhibitor.[13] Indeed rapamycin analogs (mTOR inhibitors) have been approved by the US Food and Drug Administration for the treatment of advanced PanNETs.[13,14] The mechanisms leading to PI3K-AKT-mTOR pathway activation in PanNETs, however, are not fully understood and data are conflicting. Corbo et al[15] analyzed 36 primary PanNETs and found no mutations in 6 key genes in the mTOR pathway

Accepted for publication September 27, 2013.

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The authors have no relevant financial interest in the products or companies described in this article.

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(*AKT2, PIK3CA, RPS6K1, STK11, PDPK1,* and *FRAP1-mTOR*). On the other hand, Jiao et al[11] analyzed 68 PanNETs and identified mutations in the genes involved in the mTOR pathway, including *PIK3CA* (1.4%), *PTEN* (7.3%), and *TSC2* (8.8%). Phosphatase and tensin homologue (PTEN) functions primarily as a crucial negative regulator of the PI3K-AKT-mTOR pathway by dephosphorylating phosphatidylinositol-3,4,5-trisphosphate (PIP3). Loss of PTEN function thus leads to activation of the PI3K-AKT-mTOR pathway, resulting in cell survival and proliferation.[16,17] In PanNETs, low PTEN has been shown to be associated with worse outcome. In a tissue microarray–based study of 136 well-differentiated PanNETs, Missiaglia et al[10] have shown that *PTEN* was one of the down-regulated genes in PanNETs, using global gene expression; and low PTEN protein expression, by immunohistochemistry, has been associated with shorter time to disease progression and shorter disease-free survival. PTEN, however, also functions as a tumor suppressor independent of the PI3K-AKT-mTOR pathway.[18–20] Thus, loss of PTEN expression in PanNET may be insufficient in identifying patients with an activated PI3K-AKT-mTOR pathway targetable by mTOR inhibitors.

PanNETs have been shown to express hormone receptors[6,21–23] and there is some evidence that PTEN and hormone receptors may be functionally linked. For example, in vitro studies of breast and prostate cancer cells have shown that androgen receptor (AR) functions as a transcriptional regulator of PTEN expression by binding to the promoter region of the *PTEN* gene via androgen response elements in a tissue-dependent manner.[24] Although evidence suggests that the *PTEN* promoter region does not contain estrogen response elements,[25] in vitro studies using breast cancer cells have shown that estrogen receptor (ER) regulates PTEN expression at the posttranscriptional level by inhibiting degradation.[26] In vitro studies of breast cancer cell lines have shown that progesterone receptor (PR) expression is down-regulated by insulin-like growth factor 1 (IGF-1) via PI3K-AKT-mTOR activation, independent of ER activity.[27] In addition, in vitro and in vivo studies have also shown that PR expression is regulated by PI3K-AKT pathway in endometrial cancer.[28]

The interactions between hormone receptors and PTEN have not been elucidated in PanNETs. Moreover, the importance of their combined immunohistochemical positivity, specifically PR and PTEN, as a predictor of worse outcome has not been explored. We therefore analyzed the clinicopathologic characteristics of 160 low-grade (G1) and intermediate-grade (G2) PanNETs in patients with long-term follow-up, using whole tissue sections, to determine the associations among hormone receptor (AR, ER, and PR) immunohistochemical positivity, PTEN immunohistochemical positivity, clinicopathologic features, metastasis-free survival, and overall survival.

MATERIALS AND METHODS

Case Selection

A retrospective review of the surgical pathology files at The University of Texas MD Anderson Cancer Center (UTMDACC), Houston, Texas, identified 160 consecutive patients with G1 and G2 PanNETs, on the basis of proliferative index (Ki-67 labeling) as defined by the WHO,[1] between 1990 and 2010, and with material available for immunohistochemical studies. Among 160 cases, most (142 cases, 89%) were resected between 2000 and 2010, while only 18 cases (11%) were resected between 1990 and 1999. The tumors included 152 primary PanNETs and 8 metastases (4 involving lymph nodes, 3 involving liver, and 1 involving soft tissue). Among 152 patients with resected primary PanNETs, 37 also had material available at the metastatic site for immunohistochemical analyses, including lymph node (22), liver (11), both liver and lymph node (3), and spleen (1). High-grade pancreatic neuroendocrine carcinomas were specifically excluded, as these typically have a more predictable clinical outcome. The study was approved by the Institutional Review Board of UTMDACC.

Hematoxylin-eosin–stained slides prepared from routinely processed partial pancreatectomy specimens (including pancreaticoduodenectomy, distal pancreatectomy, and enucleation) and/or resected metastases fixed in 10% buffered formalin were reviewed in all cases, as well as the corresponding surgical pathology reports. The pathologic tumor stage was determined according to the pancreatic tumor staging system based on the 7th edition criteria of the *AJCC Cancer Staging Manual*,[8] namely, stage I, tumor limited to the pancreas, irrespective of size; stage IIA, tumor extending beyond the pancreas but without involvement of the celiac axis or the superior mesenteric artery; stage IIB, regional lymph node metastasis; stage III, tumor involvement of the celiac axis or superior mesenteric artery; and stage IV, distant metastasis.

Immunohistochemistry for Ki-67, AR, ER, PR, and PTEN

By immunohistochemistry, screening of potential proteins (AR, ER, PR and PTEN) were performed on tissue microarrays (TMAs) of 80 PanNETs. Immunohistochemical staining of proteins which exhibited significant staining in TMAs (PR and PTEN) were, then, performed on 5 um thick formalin-fixed (10% buffered formalin), parafin-embedded whole tumor sections from 160 PanNETs. Standard avidin-biotin complex technique was used with antibodies to Ki-67 (1:100; clone MIB-1, Dako, Carpinteria, California), AR (1:30; clone AR441, Dako), ER (1:35; clone 6F11, Novocastra, Buffalo Grove, Illinois), PR (1:200; clone PgR1294, Dako), and PTEN (1:100; clone 6H2.1, Dako). Quantification of the proliferative index by Ki-67 immunohistochemistry was performed by using computer-assisted image analysis with Adobe Photoshop CS4 software (San Jose, California) count tool and counting the number of Ki-67 labeled and hematoxylin-stained (negative) nuclei in a photomicrograph equaling 1 HPF (×400). The highest percentage of Ki-67–labeled nuclei in 1 or more HPFs, counting at least 500 tumor cells,[1] was used to determine the grade. G1 PanNETs had Ki-67 labeling of up to 2.99%, while G2 PanNETs had Ki-67 labeling of 3.0% to 20.0%. High-grade pancreatic neuroendocrine carcinomas, those with greater than 20.0% Ki-67 labeling, were excluded. Nuclear staining for PR in 1% or more of the whole tumor section was considered positive, while those cases with staining in less than 1% of the section were considered negative, a cutoff used in breast cancer[29,30] and one previous study on PanNET.[6] PTEN expression was recorded as follows: positive, both cytoplasmic and nuclear staining intensity in greater than 10% of tumor cells was equal to that of stromal cells (internal positive control); low, at least 90% (ie, 90%–100%) of tumor cells showed weak or faint cytoplasmic and nuclear staining, compared to stromal cells; and negative, at least 90% (ie, 90%–100%) of tumor cells showed complete loss of PTEN immunohistochemical staining, as outlined in a validation study of PTEN immunohistochemistry by Sangale et al.[31]

Patient Follow-up and Survival

Clinical data and follow-up information through December 2011 were extracted from patient medical records and by review of the US Social Security Index, when necessary. Functionality was defined as documented increase in blood hormone levels and concomitant constellation of typical signs and symptoms, as defined by the WHO.[1] In patients who presented without distant metastasis (stage I and II disease), metastasis-free survival was calculated as the time from the date of diagnosis to the date of first occurrence of distant metastasis by post-surgical radiographic imaging studies (in patients who developed metastasis) or to the date of last follow-up (in patients without distant metastasis). Patients who remained alive and disease-free were censored at the

Table 1. Clinicopathologic Characteristics of 160 Patients With Pancreatic Neuroendocrine Tumors

Characteristics	No. (%)
Age, y	
Mean, 55 (range, 18–79)	
Sex	
Female	63 (39)
Male	97 (61)
Associated syndrome	
MEN1	11 (7)
Other[a]	2 (1)
Functional tumors	25 (16)
Tumor size, cm	
Mean, 4.4	
Median, 2.5	
Range, 0.8–20	
WHO grade	
Low (G1)	89 (56)
Intermediate (G2)	71 (44)
AJCC stage	
IA	27 (17)
IB	13 (8)
IIA	33 (21)
IIB	35 (22)
III	0 (0)
IV	52 (33)
Progesterone receptor	
Positive (>1%)	121 (76)
Negative (0%)	39 (24)
PTEN expression	
Positive	144 (90)
Low (>90% weak staining)	16 (10)
Follow-up, median (range), mo	57.5 (2.4–357)
Metastasis-free survival, median time	Not reached
No. of events	24
No. of patients (stage I-II)	103
Overall survival, median time	Not reached
No. of events	25
No of patients	155

Abbreviations: AJCC, American Joint Committee on Cancer; MEN1, multiple endocrine neoplasia type 1; PTEN, phosphatase and tensin homologue; WHO, World Health Organization.

[a] Other: tuberous sclerosis (1 case) and von Hippel–Lindau syndrome (1 case).

time of last follow-up. Overall survival was calculated as the time from the date of diagnosis to the date of death or the date of last follow-up (if death did not occur). Patients who did not die were censored at the time of last follow-up. Of note, 5 patients died from perioperative complications and were excluded from subsequent survival analyses.

Statistical Methods

χ^2 Analysis or Fisher exact test was used to compare categorical data, and analysis of variance was used to compare continuous variables. Survival curves were constructed by using Kaplan-Meier method and log-rank test was used to determine differences between groups. Univariate Cox proportional hazards regression models were fit to determine the association between survival and clinical and pathologic characteristics. Significant factors in the univariate models were included in a multivariate Cox proportional hazards regression model to assess the association between survival and clinical and pathologic characteristics, adjusting for other factors. A 2-sided significance level of .05 was used for all statistical tests. All statistical analyses were performed by using SAS 9.3 for Windows (2011; SAS Institute Inc, Cary, North Carolina).

RESULTS

Clinical and Pathologic Features

The clinical and pathologic features of 160 patients with PanNET are summarized in Table 1. The patients' mean age was 55 years (range, 18–79 years) and 97 (61%) were males. Eleven (7%) patients had multiple endocrine neoplasia type 1, one had von Hippel–Lindau syndrome, and one had tuberous sclerosis. Twenty-five (16%) PanNETs were functional, including 17 insulinomas, 5 gastrinomas, and 3 glucagonomas. Among patients with sporadic, nonfunctional tumors, approximately half (48%) presented with symptoms including abdominal, back, or flank pain in 57 cases, gastrointestinal symptoms (diarrhea, nausea, vomiting, melena) in 9 cases, obstructive jaundice in 7 cases, and weight loss in 4 cases. In 42 patients (26%), however, the pancreatic tumor was incidentally found during computed tomography scan or abdominal ultrasonography for various other reasons.

Mean tumor size was 4.4 cm and median tumor size was 3.5 cm (standard deviation, 3.2; standard error of mean, 0.3; range, 0.8–20 cm). According to the WHO classification standards,[1] 56% (89 cases) were G1 PanNETs and 44% (71 cases) were G2 PanNETs. As based on the AJCC (7th edition) staging manual,[8] 40 patients (25%) had stage I disease (stage IA [27] and stage IB [13]), 68 patients (43%) had stage II disease (stage IIA [33] and stage IIB [35]), and 52 patients (33%) had stage IV disease. No patients had stage III disease, as those patients would have tumors considered to be unresectable. Liver was the most common site of distant metastasis at presentation (44 of 52 patients, 85%).

Immunohistochemical Expression of Hormone Receptors and PTEN

Immunohistochemistry for AR, ER, and PR was first assessed in a subset of PanNETs by using tissue microarrays (n = 80). In the tumors analyzed, no tumors had any detectable ER staining and only 1 tumor showed weak to moderate AR nuclear staining in 75% of tumor cells (data not shown). Therefore, additional studies with these 2 hormone receptors were not pursued. On the other hand, nuclear PR staining was present in adjacent islets of Langerhans, which served as internal control, and in most tumors included in TMAs (results of staining in TMAs are not shown). We therefore performed PR immunohistochemistry in whole tissue sections from resected PanNETs from 160 patients (Figure 1). Nuclear PR positivity was seen in 121 tumors (76%), with 17 of 121 cases (14%) displaying 1% to 10% of tumor cells' staining, 32 cases (26%) displaying 10% to 50% staining, and most PanNETs (72 cases, 60%) displaying greater than 50% of tumor cells' staining. In PR-negative PanNETs, weak and very focal (<1%) staining was present in 19 of 38 (50%) tumors, while the remaining 19 cases had completely negative PR immunohistochemistry. Absent PR staining was significantly associated with larger tumor size ($P = .01$), nonfunctional PanNETs ($P = .01$), and advanced AJCC stage ($P = .002$) but not age, sex, associated syndrome, and WHO grade.

PTEN cytoplasmic and nuclear staining were strong and diffuse in stromal and endothelial cells, as well as islets of Langerhans (Figure 1) in adjacent normal pancreatic tissue,

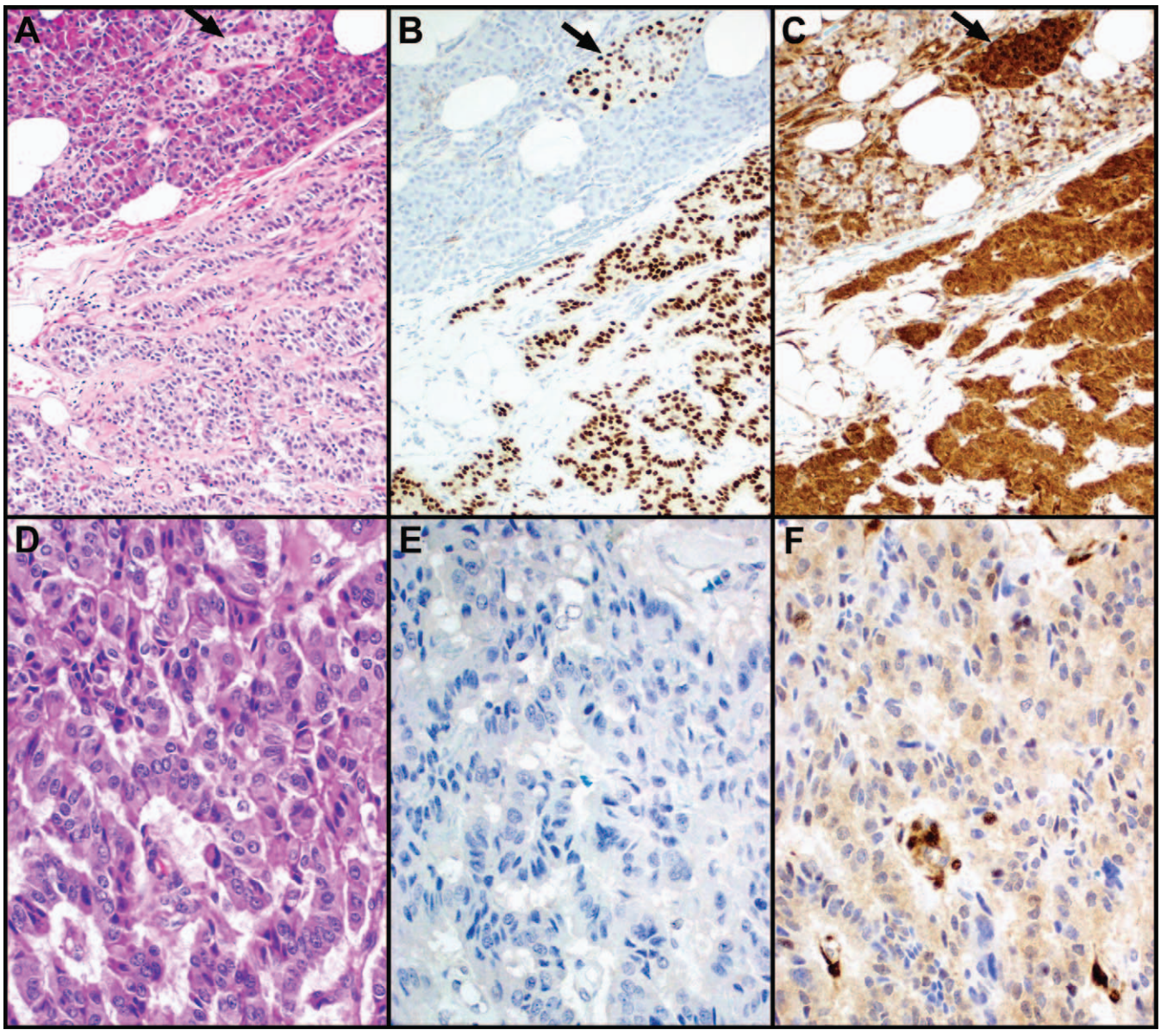


Figure 1. *A through C. Pancreatic neuroendocrine tumor (A) expressing both progesterone receptor (PR) (B) and phosphatase and tensin homologue (PTEN) (C). Of note, islets of Langerhans (A, arrow) also express both PR (B, arrow) and PTEN (C, arrow). D through F, Pancreatic neuroendocrine tumor (D) with absent PR (E) expression and low PTEN (F) expression. There is strong PTEN staining in stromal cells, which serve as internal positive control (F) (hematoxylin-eosin, original magnifications ×200 [A] and ×400 [D]; original magnifications ×200 [B and C] and ×400 [E and F]).*

and served as internal control; however, staining in PanNETs was highly variable. A total of 126 PanNETs (79 %) displayed strong and diffuse (>90%) cytoplasmic PTEN staining, 71 (44%) of which showed variable nuclear staining, ranging from approximately 20% to 100% of tumor nuclei positive for PTEN. Two cases (1%) had strong and diffuse cytoplasmic staining in 50% to 75% of tumor cells with weak to moderate staining in the remaining tumor. Six cases had diffuse cytoplasmic PTEN staining with moderate intensity, 3 with and 3 without nuclear staining. Seven (4%) cases had greater than 10% of tumor cells staining with a mosaic pattern of moderate and strong cytoplasmic staining, with and without nuclear staining. These tumors, 139 PanNETs, were categorized as PTEN positive. Sixteen cases (10%) displayed weak cytoplasmic (without nuclear) staining in at least 90% of tumor cells; these PanNETs were categorized as PTEN low. Complete loss of PTEN expression in at least 90% of tumor cells was not present in any tumor; however, heterogeneous staining with partial (5%–50%) loss of PTEN expression, accompanied by moderate to strong staining in the remaining tumor cells, was observed in 5 cases. These cases were also considered positive for the purpose of data analysis, as they did not meet the 90% cutoff. Overall, there were 144 PTEN-positive and 16 PTEN-low tumors. We found no association between PTEN expression and age, sex, associated syndrome, functionality, tumor size, WHO grade, and AJCC stage.

Given evidence from in vitro studies on breast and endometrial cancer cell lines of PI3K-AKT-mTOR pathway

Table 2. Clinicopathologic Characteristics of Patients With Pancreatic Neuroendocrine Tumors According to Combined Progesterone Receptor (PR)–Phosphatase and Tensin Homologue (PTEN) Expression

Characteristics	PR and PTEN Positive (N = 110) No. (%)	Either PR or PTEN Positive (N = 45) No. (%)	PR Negative and PTEN Low (N = 5) No. (%)	*P* Value
Age, y				
Mean ± SEM	55 ± 1.2	54 ± 1.9	57 ± 5.8	.88
Sex				
Female	40 (63)	22 (35)	1 (2)	.27
Male	70 (72)	23 (24)	4 (4)	
Syndromic patients	10 (83)	2 (17)	0 (0)	.67
Functional tumors	21 (84)	4 (16)	0 (0)	.22
Tumor size				
Mean ± SEM	4.0 ± 0.3	5.1 ± 0.4	6.3 ± 1.8	.07
WHO grade				
Low (G1)	63 (71)	25 (28)	1 (1)	.28
Intermediate (G2)	47 (66)	20 (28)	4 (6)	
AJCC stage				
I-IIA	58 (80)	14 (19)	1 (1)	.047
IIB	23 (66)	11 (31)	1 (3)	
IV	29 (56)	20 (38)	3 (6)	

Abbreviations: AJCC, American Joint Committee on Cancer; PR, progesterone receptor; PTEN, phosphatase and tensin homologue; SEM, standard error of mean; WHO, World Health Organization.

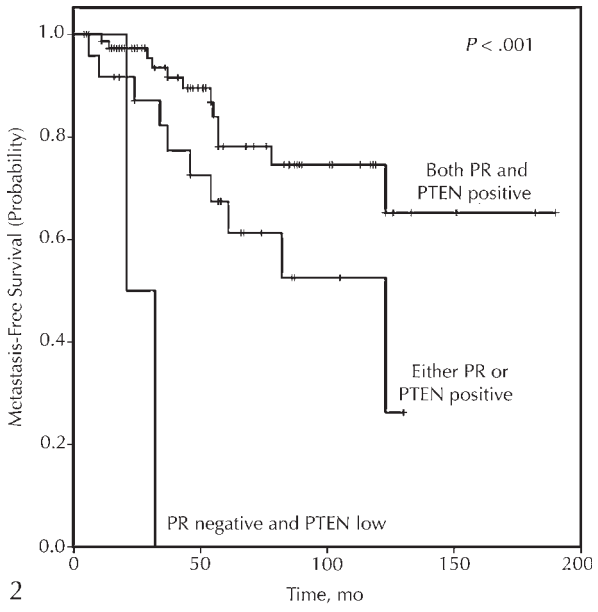
regulation of PR expression, we looked at the association of PR and PTEN and the prognostic significance of combined PR-PTEN immunohistochemical expression in PanNETs in our study. Although there was no statistically significant association between PR and PTEN expression, most PanNETs expressed both PR and PTEN (110 cases, 69%; Figure 1, A through C), while 34 cases (21%) had a PR-negative–PTEN-positive immunohistochemical profile, 11 cases (7%) had a PR-positive–PTEN-low profile, and 5 cases (3%) had a PR-negative–PTEN-low profile (Figure 1, D through F). The PR-negative–PTEN-positive group and PR-positive–PTEN-low group were combined into 1 group for subsequent analyses. In all, the PR-positive–PTEN-positive group contained 110 cases (69%), the group that was either

Table 3. Univariate and Multivariate Analyses of Clinicopathologic Factors Predicting Metastasis-Free Survival for Patients With Stage I-II Pancreatic Neuroendocrine Tumors

Characteristics	N	Univariate Analysis HR	95% CI	*P* Value	Multivariate Analysis HR	95% CI	*P* Value
Age (continuous variable)	103	1.0	1.0–1.1	.13			
Sex							
Female (ref)	44						
Male	59	0.9	0.4–2.0	.83			
Tumor size (continuous variable)	102	1.1	1.0–1.3	.01	1.1	1.0–1.3	.049
WHO grade							
Low (G1, ref)	64						
Intermediate (G2)	39	2.7	1.2–6.2	.02	3.3	1.3–8.2	.01
AJCC stage							
IA-IIA (ref)	69						
IIB	34	4.5	1.9–10.6	<.001	3.4	1.4–8.2	.006
PR expression							
Positive (ref)	85						
Negative	18	2.1	0.9–4.8	.10			
PTEN expression							
Positive (ref)	93						
Low	10	3.9	1.6–9.9	.004			
Combined PR-PTEN							
Both PR and PTEN positive (ref)	77			.001			.007[a]
Either PR or PTEN positive	24	2.3	1.0–5.4	.05	1.8	0.7–4.4	.19
PR negative and PTEN low	2	21.0	4.2–106.0	<.001	13.7	2.5–73.7	.002

Abbreviations: AJCC, American Joint Committee on Cancer; CI, 95% confidence interval; HR, hazard ratio; PR, progesterone receptor; PTEN, phosphatase and tensin homologue; ref, reference; WHO, World Health Organization.

[a] Overall group *P* value.



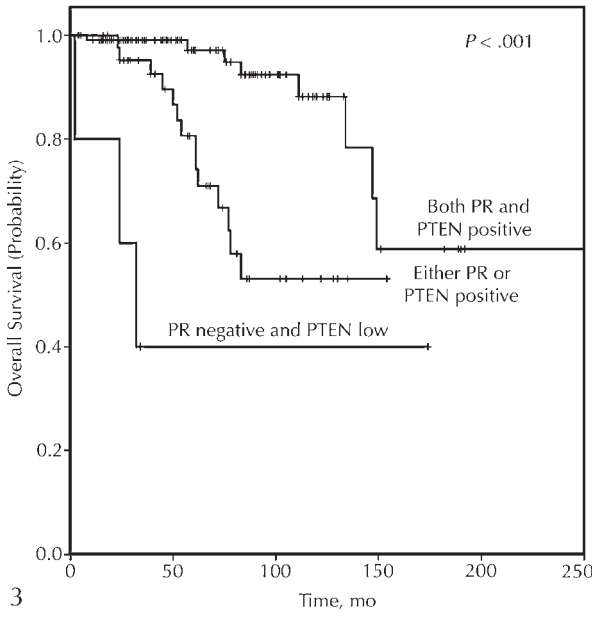


Figure 2. *Kaplan-Meier estimate of metastasis-free survival in patients with pancreatic neuroendocrine tumors (PanNETs). Patients with progesterone receptor (PR)–positive and phosphatase and tensin homologue (PTEN)–positive PanNETs had the longest metastasis-free survival.*

Figure 3. *Kaplan-Meier estimate of overall survival in patients with pancreatic neuroendocrine tumors (PanNETs). Patients with progesterone receptor (PR)–positive and phosphatase and tensin homologue (PTEN)–positive PanNETs had the best overall survival.*

PR or PTEN positive contained 45 cases (28%), and the PR-negative–PTEN-low group contained 5 cases (3%). PanNETs expressing both PR and PTEN were significantly associated with lower AJCC stage ($P < .05$) but not with age, sex, associated syndrome, functionality, tumor size, and WHO grade (Table 2).

The 5 patients with PR-negative–PTEN-low PanNETs were all males with a mean age of 57 years (range, 47–65 years). None of the patients had an associated syndrome. None of the tumors were functional. The average tumor size was 6.3 cm (range, 2.5–11 cm) and most (4 of 5, 80%) were G2 (intermediate grade). Three patients (60%) presented with stage IV disease, the remaining 2 patients had stage II (1 with stage IIA and 1 with stage IIB) disease and developed metastasis at a later time. At last follow-up, 3 patients died of disease while 2 patients were alive with disease.

Among 37 patients with both primary and metastatic tumors, 31 (84%) had concordant combined PR-PTEN immunohistochemical expression profile. For 3 concordant PR and discordant PTEN profiles, 2 cases showed low PTEN expression in the primary tumor, while the regional lymph node metastasis expressed PTEN, and 1 case showed PTEN expression in the primary site but low expression in the metastatic site. For 2 concordant PTEN but discordant PR profiles, 1 primary PanNET exhibited 1% PR staining while the metastatic site exhibited complete absence of PR expression, and the other primary PanNET exhibited 70% PR staining while the regional lymph node metastasis showed complete absence of PR expression. Finally, 1 case had a PR-positive and PTEN-positive primary tumor but a PR-negative and PTEN-low liver metastasis. For all discordant cases, the immunohistochemical profile of the primary tumor was used for subsequent analyses.

Metastasis-Free Survival in Patients With Stage I and II Disease

Among 108 patients with stage I and II disease, 24 patients (22%) had distant metastasis at follow-up, 21 of whom had metastasis to the liver (88%). The median metastasis-free survival time had not been reached owing to insufficient (<50%) events. By univariate analysis, tumor size ($P = .01$), AJCC stage ($P < .001$), WHO grade ($P = .01$), PTEN immunohistochemistry ($P = .004$), and combined PR-PTEN immunohistochemistry ($P < .001$) were significantly associated with metastasis-free survival (Table 3). Patients with stage IIB disease, intermediate grade (G2) tumors, and PTEN-low immunohistochemical profile had a significantly shorter metastasis-free survival (median survival time: 78, 123, and 34 months, respectively), while median survival for their respective counterparts had not been reached. Of note, the shortest metastasis-free survival was observed in patients with PR-negative and PTEN-low PanNETs (Figure 2). Median metastasis-free survival was 27 months for these patients compared to patients with PanNETs positive for either PR or PTEN (median survival of 124 months); median survival for patients with PanNETs staining for both PR and PTEN had not been reached ($P < .001$). By multivariate analysis using a model with tumor size, AJCC stage, WHO grade, and combined PR-PTEN immunohistochemistry, all factors remained significantly associated with metastasis-free survival (Table 3).

Overall Survival

The median length of follow-up was 57 months and ranged from 2 to 357 months. Approximately half of the patients (79 cases, 49%) were alive without evidence of disease at last follow-up, 51 patients (32%) were alive with metastatic disease (including 33 patients [21%] who presented with distant metastasis and 18 patients [11%] who had distant metastasis at a later time), 25 patients (16%) died of disease, and 5 patients (3%) died from perioperative complications. The median overall survival for all patients had not been reached. The estimated 3-, 5- and

Table 4. Univariate Analysis of Clinicopathologic Factors Predicting Overall Survival for Patients With Pancreatic Neuroendocrine Tumors

Characteristics	N	HR	95% CI	*P* Value
Age (continuous variable)	155	1.0	1.0–1.0	.67
Sex				
Female (ref)	63			
Male	92	1.0	0.5–2.2	.99
Functional tumors				
No (ref)	131			
Yes	24	0.9	0.3–3.1	.88
Tumor size (continuous variable)	152	1.1	1.0–1.2	.15
WHO grade				
Low (G1, ref)	86			
Intermediate (G2)	69	1.5	0.7–3.4	.30
Lymph node metastasis				
No (ref)	86			
Yes	68	3.3	1.4–7.8	.008
AJCC stage IV disease				
No (ref)	104			
Yes	51	6.4	2.5–16.0	<.001
PR expression				
Positive (ref)	116			
Negative	39	4.4	2.0–9.8	<.001
PTEN expression				
Positive (ref)	140			
Low	15	2.9	1.1–7.8	.03
Combined PR-PTEN				
Both PR and PTEN positive (ref)	106			.001[a]
Either PR or PTEN positive	44	4.4	1.8–10.6	<.001
PR negative and PTEN low	5	9.1	2.3–36.0	.002

Abbreviations: AJCC, American Joint Committee on Cancer; CI, 95% confidence interval; HR, hazard ratio; PR, progesterone receptor; PTEN, phosphatase and tensin homologue; ref, reference; WHO, World Health Organization.

[a] Overall group *P* value.

10-year survival rates were 96%, 89%, and 74%, respectively.

Results of univariate analyses of clinicopathologic factors predicting survival are summarized in Table 4. Our study could not evaluate the importance of AJCC stage as a predictor of overall survival since patients with stage I and IIA disease did not have an event (invalid Wald test). However, the presence of distant metastasis at diagnosis (stage IV versus stage I-II disease; hazard ratio [HR] = 6.4, 95% confidence interval [CI] = 2.5–16.0, $P < .001$) and lymph node metastasis, irrespective of stage (HR = 3.3, CI = 1.4–7.8, $P = .008$) were significantly associated with worse overall survival. In patients with stage IV disease, the median overall survival was 111 months, whereas the median survival had not been reached for those patients with stage I-II disease ($P < .001$). Median overall survival for patients who presented with lymph node metastasis (n = 18), irrespective of stage, was 149 months, whereas the median survival had not been reached for those patients without lymph node metastasis ($P = .005$).

Moreover, absent PR staining (HR = 4.4, CI = 2.0–9.8, $P < .001$), low PTEN staining (HR = 2.9, CI = 1.1–7.8, $P = .03$), either PR- or PTEN-positive immunohistochemical profile (HR = 4.4, CI = 1.8–10.6, $P < .001$), and PR-negative and PTEN-low immunohistochemical profile (HR = 9.1, CI = 2.3–36.0, $P = .002$) were significantly associated with worse overall survival. Median overall survival time was 83 months for patients with PR-negative tumors, while median time for patients with PR-positive tumors had not been reached. Median times for patients when stratified by PTEN immunohistochemical profile had also not been reached. Of note, the shortest median survival time was observed in patients with PR-negative and PTEN-low PanNETs. Median overall survival for these patients was 32 months, whereas median overall survival had not been reached for patients with PanNETs staining for both PR and PTEN, or for patients staining for either PR or PTEN ($P < .001$, Figure 3).

Two multivariate models were used to determine the importance of clinicopathologic features as predictors of overall survival, controlling for other significant factors (Table 5). In the first model, PR and PTEN immunohistochemistry were assessed separately, while in the second model, combined PR-PTEN immunohistochemical profile was assessed as 1 factor. Progesterone receptor immunohistochemistry alone (negative versus positive; HR = 3.1, CI = 1.4–7.1, $P = .007$), as well as PTEN immunohistochemistry alone (low versus positive; HR = 6.8, CI = 2.1–21.6, $P = .001$), were significantly associated with overall survival. When combined, PR and PTEN staining was also significantly associated with overall survival ($P < .001$). In addition, the presence of distant metastasis but not lymph node metastasis remained significantly associated with overall survival in both models.

Table 5. Multivariate Analyses of Clinicopathologic Factors Predicting Overall Survival for Patients With Pancreatic Neuroendocrine Tumors

Characteristics	Model No. 1				Model No. 2			
	N	HR	95% CI	*P* Value	N	HR	95% CI	*P* Value
Lymph node metastasis								
No (ref)	86							
Yes	68	1.9	0.7–5.1	.21				
AJCC stage IV disease								
No (ref)	103							
Yes	51	6.6	2.2–19.4	<.001				
PR expression								
Positive (ref)	115							
Negative	39	3.1	1.4–7.1	.007				
PTEN expression								
Positive (ref)	139							
Low	15	6.8	2.1–21.6	.001				
Lymph node metastasis								
No (ref)					86			
Yes					68	1.8	0.6–5.0	.26
AJCC stage IV disease								
No (ref)					103			
Yes					51	6.0	2.1–17.0	<.001
Combined PR-PTEN								
Both PR and PTEN positive (ref)					105			<.001[a]
Either PR or PTEN positive					44	3.5	1.4–8.9	.009
PR negative and PTEN low					5	19.3	4.5–82.9	<.001

Abbreviations: AJCC, American Joint Committee on Cancer; CI, 95% confidence interval; HR, hazard ratio; PR, progesterone receptor; PTEN, phosphatase and tensin homologue; ref, reference.

[a] Overall group *P* value.

Treatment of Patients With PanNET

Among patients with PanNET in our study, treatment varied considerably. Somatostatin analog was given to 7 patients before resection and 1 patient after resection. Forty-nine patients received chemotherapy, 45 in the neoadjuvant setting, 2 in the adjuvant setting, and another 2 received chemotherapy before and after resection. Five patients received everolimus (mTOR inhibitor), and 6 patients received radiation therapy. Since the most important factor guiding treatment of patients was tumor stage at presentation, our study population was subdivided according to stage for subsequent survival analyses comparing different treatment regimens (somatostatin analog, chemotherapy, and radiation therapy). Among patients with stage I-IIA disease, none received additional treatment. Among patients with stage IIB or stage IV disease, there was no difference in metastasis-free and overall survival for patients who received additional therapy, compared to those who did not receive additional therapy (data not shown).

Among patients with recurrent/metastatic PanNETs, resection of the recurrent/metastatic tumor was performed in 24 patients and chemotherapy and/or everolimus was given to 58 patients. Patients who underwent resection had a significantly better overall survival than those who did not. Median overall survival was 111 months for patients who did not undergo resection, while median overall survival had not been reached for patients who underwent resection ($P = .009$). The addition of chemotherapy and/or everolimus did not affect overall survival (data not shown).

COMMENT

Pancreatic neuroendocrine tumors have varying behavior, ranging from indolent to aggressive. The proliferative rate (WHO grade) and extent of disease (tumor stage) have emerged as the most important factors that predict aggressive behavior.[3–7] In this study, we showed that combined PR-PTEN immunohistochemistry is also prognostically important for low- and intermediate-grade PanNETs.

Although it is difficult to compare our data with those of previous studies on prognosis in patients with PanNETs,[3–7] owing to the changes made to the criteria used by the current WHO grading and AJCC staging systems and the different parameter cutoffs used for analysis, our findings confirm the prognostic value of the 2010 WHO grading criteria and AJCC (7th edition) staging system in PanNETs. In our study, after controlling for other significant factors, both the WHO grade and AJCC stage remained significantly associated with metastasis-free survival in patients who presented with stage I and II disease after resection. Although the significance of AJCC stage in predicting overall survival could not be evaluated in our patient population owing to insufficient power, our study showed that the presence of distant metastasis significantly correlated with worse overall survival and highlights the importance of clinical and pathologic evaluation for the presence of distant metastasis at the time of diagnosis in risk stratification. Our study also highlights the importance of aggressive resection of metastatic/recurrent PanNETs. Patients who underwent resection had a significantly better overall survival than patients who did not undergo resection

of their recurrent/metastatic tumors. Our study, however, failed to demonstrate the prognostic significance of the WHO grading system in predicting overall survival using the current criteria, supporting the need for additional prognostic factors in PanNETs.

Activation of the PI3K-AKT-mTOR pathway in PanNETs has been shown to play an important role in a subset of PanNETs, especially those associated with advanced disease, as evidenced by the improvement in progression-free survival seen in patients with advanced PanNETs treated with mTOR inhibitors.[13] Although the mechanisms by which the mTOR pathway is activated in PanNETs are still poorly understood, PTEN has been shown to be a crucial negative regulator of the PI3K-AKT-mTOR pathway. While its role in PanNET tumorigenesis is still unclear and somatic mutations in *PTEN* are rare in PanNETs (3%[32] to 7%[11]), there is growing evidence suggesting that other mechanisms can cause loss of PTEN function. These include epigenetic silencing through aberrant promoter methylation,[33] transcriptional inhibition,[34] and microRNA regulation.[35] Hence, assessing PTEN protein expression by immunohistochemistry may be more accurate than *PTEN* gene sequencing for determining PTEN functional status in a tumor,[36] and PTEN immunohistochemical assessment has been found to be highly reproducible.[37]

Low PTEN immunohistochemical staining in PanNETs has been shown to be significantly associated with worse survival. Missiaglia et al[10] analyzed 136 well-differentiated PanNETs by using tissue microarrays (TMAs) and found that low PTEN positivity correlated with a shorter time to disease progression and shorter disease-free survival. Our study has validated the importance of PTEN immunohistochemistry in predicting metastasis-free and overall survival on whole tumor sections. Moreover, when adjusting for other significant factors, we found that PTEN immunohistochemistry remained significantly associated with overall survival. Krausch et al[38] analyzed 38 PanNETs for PTEN staining by using greater than 10% as cutoff for positive expression (similar to our cutoff). They found that low PTEN staining was associated with advanced WHO grade and TNM stage; however, their findings did not reach statistical significance, most likely due to insufficient power. With a larger study population, our study did not find significant associations between PTEN staining and WHO grade and AJCC stage. The question remains whether PTEN expression may act as a surrogate marker for activated PI3K-AKT-mTOR pathway in PanNETs given its multitude of functions independent of this pathway.[18–20]

The importance of hormone expression (PR, AR, and ER) in predicting survival for patients with PanNET is unclear. With regard to AR and ER, our study on TMAs suggests that immunohistochemical staining for these 2 hormone receptors may not have utility in predicting prognosis in PanNETs. Although Alabraba et al[23] found ER (α) positivity in 10 of 25 insulinomas (40%), our study (n = 80) and that of Viale et al[22] (n = 96) found no ER staining in the tumors analyzed, while Arnason et al[21] found only weak and focal staining in 5 of 40 tumors (12%), with no tumors exhibiting strong nuclear staining in their study. To date, no study has reported AR positivity in PanNETs and we found positive nuclear staining for AR in only 1 tumor in our study (n = 80). The utility of PR immunohistochemistry as a prognostic factor is conflicting. Hochwald et al[6] evaluated 87 patients with PanNETs who had undergone curative resection and found that PR staining did not impact disease-free ($P = .79$) or disease-specific ($P = .28$) survival by univariate analysis, using the same 1% cutoff as in our study. On the other hand, Viale et al[22] analyzed 96 PanNETs and showed that absent PR staining is significantly associated with the presence of metastases, large vessel invasion, and extension into adjacent organs ($P < .001$). Pelosi et al[39] analyzed 54 PanNETs and showed that absent PR staining correlated with shorter survival ($P = .01$) by univariate analysis. Similar to the 2 latter studies, we found that patients with PR-negative PanNETs had a significantly worse overall survival that remained statistically significant after controlling for other factors. Progesterone receptor immunohistochemistry, however, was not a significant predictor of metastasis-free survival in our study. These findings highlight the shortcoming of PR immunohistochemistry, alone, as a prognostic factor in patients with PanNET.

Moreover, the role of PR expression in PanNET tumorigenesis has not been elucidated; however, evidence from breast[27,40] and endometrial[28] carcinomas suggests that PR expression may be regulated by the PI3K-AKT-mTOR pathway. In vitro studies of breast cancer cell lines by Cui et al[27] have shown that *PR* expression is down-regulated by IGF-1 via PI3K-AKT-mTOR activation, independent of estrogen receptor activity. Breast cancer cells treated with IGF-1 showed decreased *PR* messenger RNA levels through inhibition of *PR* promoter activity. Blockade of the PI3K-AKT-mTOR pathway using 1L-6-hydroxymethyl-*chiro*-inositol-(R)-2-*O*-methyl-3-*O*-octadecylcarbonate (HIMOC, AKT inhibitor), rapamycin (mTOR inhibitor), or LY294002 (PI3K inhibitor) rescued IGF-1 down-regulated PR expression to control levels. Moreover, breast cancer cells with constitutively active *AKT* showed decreased PR expression. A study of 131 patients with breast cancer by Tokunaga et al[40] has shown that loss of heterozygosity at the *PTEN* locus leads to activation of the AKT pathway and in turn is inversely correlated with PR expression. In vitro and in vivo studies by Gu et al[28] have also shown that PR expression is regulated by PI3K-AKT pathway in endometrial cancer cells lines. In progestin-resistant (ie, markedly downregulated PR expression) endometrial cancer cells, inhibition of the PI3K-AKT pathway (via LY294002) caused upregulation of PR expression by 180%, compared to control ($P > .05$). Female mice with progestin-resistant xenografts treated with LY294002 showed reversal of progestin resistance and had a 59% reduction in tumor volume compared to 38% reduction in controls ($P > .05$) when treated with progestin. Taken together, these studies suggest that the PI3K-AKT-mTOR pathway may be involved in PR expression regulation. Our study did not show significant association between PR and PTEN immunohistochemistry in PanNETs. It is possible that multiple pathways regulate PR and PTEN protein levels, which highlights the importance of combined PR and PTEN immunohistochemistry to potentially identify tumors with activated PI3K-AKT-mTOR pathway. In vitro and in vivo studies are required to elucidate the mechanisms driving PR and PTEN expression, their roles in the PI3K-AKT-mTOR pathway, and the importance of their expression in predicting treatment response to mTOR inhibitors in PanNETs.

Our study has shown that combined PR and PTEN immunohistochemistry is an independent predictor of metastasis-free and overall survival. Among 160 patients with G1 and G2 PanNETs in our study, we found 5 cases (3%) with both PR-negative and PTEN-low immunohistochemical profile. These patients had the shortest metastasis-free survival and overall survival, compared to those patients with PanNETs positive for PR and PTEN, independent of

other clinicopathologic factors. The 3-year and 5-year survival rates in our patient population were approximately 96% and 89%, respectively, which highlights the rarity of deaths in patients with PanNETs. The rarity of patients with PR-negative–PTEN-low PanNETs, as it predicts worst survival, is compatible with the survival rate of patients with PanNET and highlights the value of combined PR-PTEN immunohistochemistry in potentially identifying rare patients who may have poor outcome. In addition, as the immunohistochemical profiles between the primary and metastatic tumors were highly concordant (84%), assessment of these 2 markers in biopsy specimens may help in stratifying patients and guide treatment accordingly. Since only 3% of our cases have this immunohistochemical profile, additional studies with a larger PR-negative and PTEN-low patient population are needed to further validate these results.

In summary, our study of 160 patients with PanNETs showed that immunohistochemistry for PR and PTEN, in combination, may enhance our ability to predict outcome, independent of WHO grade and AJCC stage. Furthermore, PR and PTEN expression between the primary tumor and metastases is highly concordant, suggesting a role for risk stratification in patients with resected PanNETs.

This work was funded, in part, by a Stand Up to Cancer/American Association for Cancer Research Dream Team Translational Cancer Research Grant, Grant No. SU2C-AACR-DT0209 (R.R.B.) and supported by the National Institutes of Health under Ruth L. Kirschstein National Research Service Award No. 5 T32 CA009598-24 (A.M.).

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