Palladin is a Marker of Liver Metastasis in Primary Pancreatic Endocrine Carcinomas*

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Abstract. *Background: Palladin is a metastasis-associated gene regulating cell motility. The expression of palladin protein in pancreatic neuroendocrine tumors (PET) and carcinomas (PECA) is not known. Materials and Methods: A tissue microarray (TMA) of well-differentiated (WD) PETs/PECAs (AJCC 2010) and non-neoplastic, histologically normal pancreatic tissue/islets (HNPIs) was immunostained with palladin antibody and quantified using the Allred score. The results were correlated with the presence or absence of liver metastases. Results: The retrospective study included 19 males and 19 females of age 27-79 years (mean 54). Tumor size was 0.9-11.5 cm (mean 3.8). Palladin expression was cytoplasmic and/or membranous. The tumors with high palladin expression were associated with liver metastasis ($p<0.0001$). All 14 primary PECA with hepatic metastases (MP-PECAs) exhibited palladin expression whereas 14 out of 24 (58%) clinically-localized primary PET (CLP-PETs) expressed palladin ($p<0.01$) with median Allred scores of 5 (range 3-7) and 2 (range 0-6) respectively ($p<0.0001$). The mean Allred score for the HNPIs in the MP-PECAs (N=6) was higher (4.2) as compared to that in the CLP-PETs (2.5,N=11) ($p=0.23$). Conclusion: Palladin may identify primary pancreatic endocrine neoplasms with a propensity to metastasize to the liver.*

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Pancreatic neuroendocrine tumors/carcinomas (PETs/PECAs) account for 1-2% of pancreatic neoplasms and are clinically challenging (1). The clinical behavior of PET is often unpredictable. According to the current American Joint Committee on Cancer (AJCC) Staging manual (2), well-differentiated (WD) PECAs (WD-PECAs) are separated from well-differentiated PETs (WD-PETs) on the basis of gross invasion of the adjacent organs by the tumor (duodenum, stomach, spleen) and/or the presence of metastases. With the development of metastases, patient survival significantly drops and none of the currently available therapies offer cure. The discovery of molecular markers of metastases would offer newer objective criteria to determine patient prognosis and may improve our understanding of the biology of clinical progression in PET.

The metastasis-associated gene palladin has a role in cell motility and cell-cell interactions (4, 5). Palladin protein has been reported in a variety of tissues such as prostate, testis, ovary, small intestine, colon and smooth muscle (6). It is localized along the actin filaments of smooth muscle, epithelial and glial cells in a periodic punctuate pattern (4, 6). There are three isoforms of palladin (a 90 kDa, 140 kDa, and 200 kDa) of which the 90 kDa isoform is the most common and most abundant (5). Palladin is a critical well-documented component of the cytoskeleton and co-localizes with alpha-actinin along with F-actin stress fibers (5). It belongs to a small gene family that includes the Z-disc proteins myopalladin and myotilin, all of which share similar Ig-like domains (7).

Mutation in the palladin gene (P239S) has been identified in familial and sporadic forms of pancreatic cancer and the mutated protein may impair the normal organization of the actin cytoskeleton (8). It is believed that palladin functions as a proto-oncogene and its overexpression has been demonstrated in pancreatic cancer, normal appearing pancreatic tissue adjacent to cancer and in precancerous lesions (PanIN 1-3) (8). In cultured cells expressing the P239S palladin mutation, cytoskeleton changes were observed as well

Table I. *Clinicopathological characteristics of patients with primary pancreatic endocrine neoplasms with liver metastases (MP-PECA) at the time of resection (N=14).*

Clinical-pathological characteristics	
Race	Caucasian: 10
	African-American: 2
	White Hispanic: 1
	Other: 1
Age (years)	28-79 (mean: 51)
Gender	Female: 7
	Male: 7
Tumor site in pancreas	Head: 1
	Tail: 13
Tumor size (cm)	1.5-11.5 (mean: 4.6)
Extent of invasion	Localized: 9
	Peri-pancreatic extension: 5
Ki-67	<2 to 12 % (average: 4.7%)
Mitotic activity per 10 hpf	0 to 12 (mean: 4 mitoses/10 hpf)
Necrosis	Geographic: 2
	Focal: 2
	Absent: 10
Vital status	Alive: 10
	Dead: 4
Survival time (months)	21.0-118.2 (mean: 65.4)

Table II. *Clinicopathological characteristics of patients with primary pancreatic endocrine neoplasms without liver metastases (CLP-PET) at the time of resection (N=24).*

Clinical-pathologic characteristics	
Race	Caucasian: 15
	African-American: 4
	Asian Indian: 2
	Other: 2
Age (years)	27-78 (mean: 56)
Gender	Female: 12
	Male: 12
Tumor site in pancreas	Head: 11
	Body: 1
	Tail: 12
Tumor size (cm)	1.4-10 (mean: 3.4)
Extent of invasion	Localized: 19
	Peri-pancreatic extension: 4
	Duodenum: 1
Ki-67	<1 to 40% (average: 5.3%)
Mitotic activity per 10 hpf	0 to 11 (mean: 2 mitoses/10 hpf)
Necrosis	Geographic: 1
	Focal: 1
	Absent: 22
Vital status	Alive: 21
	Dead: 3
Survival time (months)	6.5 to 154.4 (mean: 53.1)

as abnormal actin bundle assembly, and an increased ability for the cells to migrate. And the overexpression of palladin may have a role in the invasive potential of pancreatic cancer cells (8). Zogopoulos G *et al*. reported only identifying the P239S palladin variant in one out of 84 pancreatic cancer cases analyzed, suggesting that the mutated variant is rare and does not account for a significant proportion of hereditary or early onset pancreatic neoplasms (9).

Palladin protein expression has been reported in a variety of human tumors including pancreatic ductal adenocarcinoma (10), breast (11), and colon (12).

Here, was studied the expression of palladin in primary PECA with hepatic metastases (MP-PECAs) as compared to clinically-localized primary PET (CLP-PETs), using Immunohistochemistry (IHC) and the tissue microarray technology.

Materials and Methods

This retrospective study was approved by the Institutional Review Board at the University of South Florida, Tampa and included 38 consecutive adult patients who had undergone surgical resection of their primary pancreatic endocrine neoplasms at the Moffitt Cancer Center between 1996 and 2008. These cases were identified by electronic search of the Anatomic Pathology database and personal consultation files of Neuroendocrine Oncologists (JS, LKK) at Moffitt Cancer Center. Poorly-differentiated neuroendocrine carcinomas and tumors less than 0.5 cm were excluded. Fourteen out of the 38 (37%) cases had synchronous liver metastases at the time of resection of the pancreatic primary, while 24 out of the 38 cases (63%) were without liver metastases.

Histopathological analysis, grading, staging and proliferative index. For each case, selected hematoxylin and eosin stained slides were reviewed by the study pathologists (EBHJ, NAN, AN) to confirm the original pathological diagnoses and to assess the following pathological criteria: tumor size, tumor extent, mitoses per 10 high power fields (HPFs), presence or absence of focal/geographic tumor necrosis, presence or absence of regional lymph node metastases and Ki-67 index. The Ki-67 index was determined as % of tumor cells showing distinct nuclear positivity by counting up to 2000 neoplastic cells in the viable areas of each tumor showing the highest nuclear labeling. The clinico-pathological data are summarized in Tables I and II. Based on the criteria established by the AJCC (2) 14 out of the 38 (37%) primary tumors were classified as WD-PECAs and 24 of 38 (63%) as WD-PETs based on the presence or absence of gross local invasion and/or synchronous liver metastases.

Custom tissue microarray (TMA). For each case, a formalin-fixed, paraffin-embedded tumor block representative of the tumor was selected and used to construct a tissue microarray. For each case five 1 mm cores of viable tumor and five 1 mm cores of adjacent non-neoplastic pancreas containing islets cells, when available, were taken and included in the microarray block. The final TMA included samples from each of the 38 selected cases.

Immunohistochemistry. Four micron thick FFPE sections from the TMA block were immunostained for palladin using rabbit anti-

Table III. *Candidate markers for prediction of hepatic metastases*. Statistical analysis.

		Hepatic metastases		
Candidate marker	Value	Present/Absent	Association	*p*-Value
Palladin*	High	14	9	<0.0001
	Low	0	15	
Ki-67 >2%	Yes	9	10	0.31
	No	5	14	
≥2 mitoses per 10 hpf	Yes	7	6	0.16
	No	7	18	
Necrosis	Present	4	2	0.17
	Absent	9	20	
Primary tumor size >2 cm	Yes	11	19	1.00
	No	3	5	
Regional lymph nodes	Present	7	7	0.72
Metastases	Absent	7	11	

*The marker value for Palladin was based on the ROC calculation. High: Allred score ≥3; Low: Allred score <3.

human palladin polyclonal antibody (dilution 1:50) (ProteinTech Inc. Chicago, IL, USA). The IHC staining was carried out using a Ventana Discovery XT automated system (Ventana Medical Systems, Tucson, AZ, USA) as per the manufacturer's protocol with proprietary reagents. Briefly, the sections were deparaffinized in the automated system with EZ Prep solution (Ventana). Enzymatic retrieval was carried out with Protease 1 solution (Ventana). The Ventana Omni UltraMap kit detection system was used and the sections were then counterstained with hematoxylin. Slides were dehydrated and cover-slipped as per standard tissue core laboratory protocol. Human cardiac muscle tissue was used as a positive control, as per the manufacturer's recommendation. The primary antibody was replaced by normal rabbit serum in the negative control slides. The staining results were expressed as low (Allred score <3) or high (Allred score ≥3) expression. The Allred score is derived from the intensity score determined by the intensity of staining in the tumor cells (0=no staining, 1=weak, 2=intermediate, 3=strong staining) and the overall proportion of tumor cells showing positive staining and (ranging from 0 to 5). The intensity and proportion scores are added to obtain a total score which ranges from 0 to 8, as outlined in a previous study by Allred *et al*. (13).

Data analysis. The Wilcoxan rank-sum test was used to test for differences in medians of the two independent tumor sets (primary pancreatic endocrine neoplasms with and without liver metastases). Associations between the variables were assessed by Chi-square or Fisher's exact test, as appropriate for cell size. Receiver operating characteristics (ROC) analysis was used to choose the cut-off point in the palladin Allred score (high *vs*. low expression) to optimize sensitivity and specificity for predicting the presence of liver metastases. The association between the presence of liver metastases and the conventional pathological criteria of malignancy (mitoses >2/10 HPF, Ki-67 proliferation index >2%, presence of tumor necrosis, primary tumor size >2cm and the presence of lymph node metastases) was determined in comparison with palladin expression (Table III).

Results

Patients and specimen characteristics. The study comprised of 19 males and 19 females, whose ages ranged from 27 to 79 years (mean age 54 (Tables 1 and II)). Twenty-six (68%) patients were Caucasian, six (16%) were African-Americans, two (5%) were Asian Indian, and four (11%) were classified as other. Overall, the size of the tumors ranged from 0.9 cm to 11.5 cm (mean size 3.8 cm). Mean tumor size for MP-PECAs was 4.5 cm and 3.3 cm for CLP-PETs. Necrosis was present in six (16%) of the cases, 3 of which showed geographic areas of necrosis and the remaining three exhibited focal necrosis. Twenty-five (66%) of the tumors were located in the tail of the pancreas, 12 (31%) were identified in the head of the pancreas, and 1 (3%) was located in the body of the pancreas. Twenty-eight (74%) of the tumors were localized to the pancreas. Peripancreatic invasion was seen in nine (23%) of the cases and one (3%) tumor invaded into the duodenum. The examined lymph nodes were free of metastases in 18 (47%) of the cases, whereas 14 (37%) of the cases demonstrated lymph node metastasis at the time of surgery. In 6 (16%) cases no lymph nodes were present in the pathological specimen (NX) (Tables III and IV). The majority of the pancreatic endocrine neoplasms in this series were T2 (50%). There was no evidence of metastasis in 63% (24) of the cases.

Palladin expression. The palladin immunostain results are summarized in Figure 1. The IHC expression of palladin in the pancreatic endocrine neoplasms studied was cytoplasmic and/or membranous (Figure 2A,B). The high expression of palladin was significantly associated with the presence of liver

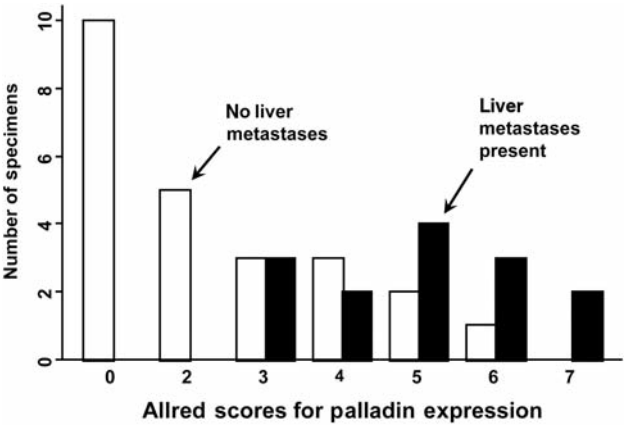

Figure 1. *Frequency distribution of Allred scores for palladin expression in pancreatic endocrine neoplasms with liver metastases (black bars, n=14 specimens total) and without liver metastases (white bars, n=24 specimens total).*

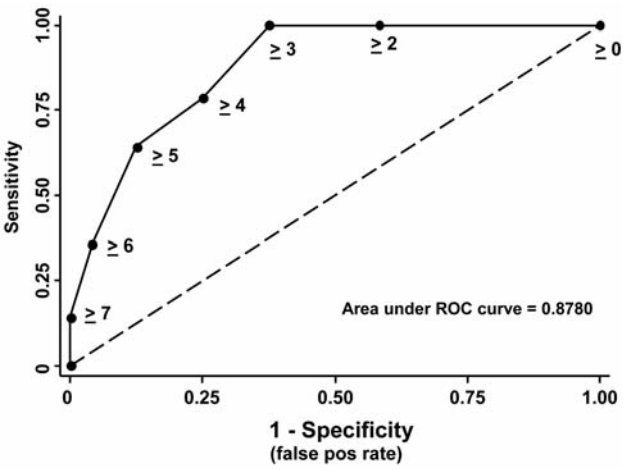

Figure 2. *Receiver operating characteristics (ROC) curve of Allred score cut-point for palladin expression in the neoplastic tissue in relation to hepatic metastases.*

metastasis ($p<0.0001$). All 14 MP-PECAs exhibited palladin expression, whereas only 14 out of the 24 (58%) CLP-PETs expressed palladin ($p<0.01$). Not only was there a significant association between hepatic metastases and palladin expression in the primary tumor, but palladin expression was also greater in tumors with hepatic metastases. The median palladin Allred score was 5 (range 3-7) in the MP-PECAs, and 2 (range 0-6) in the CLP-PETs ($p<0.0001$).

Palladin expression in non-neoplastic pancreatic islets. Non-neoplastic, histologically normal pancreatic islets (HNPIs) were available for comparison of the palladin expression with the matched tumor tissue in 6 MP-PECAs and in 11 CLP-PETs. The HNPIs from 5 out of the 6 MP-PECAs expressed palladin with a median Allred score 4 (range 0-7); whereas the HNPIs from the 6 of the 11 CLP-PETs expressed palladin with a median Allred score 3 (range 0-7) ($p=0.23$). In comparison with the HNPIs, the matched MP-PECAs showed higher expression of palladin with median Allred scores of 4 and 5 respectively. The HNPIs and matched CLP-PETs, on the other hand, showed essentially similar expression of palladin with the median Allred scores being 3 and 2 respectively. Interestingly, the HNPIs in the case of MP-PECA cases exhibited higher expression of palladin as compared to those from the CLP-PETs.

Stromal expression of palladin. Twenty-six out of the 38 (68%) pancreatic endocrine neoplasms expressed palladin also within the intratumoral stroma (Figure 3). Stromal palladin expression was generally diffuse rather than focal. Interestingly, the presence of hepatic metastases was independent of stromal expression of palladin ($p=0.47$).

Prediction of liver metastases based on palladin. The ROC curve of palladin expression in the primary pancreatic endocrine tumor tissue the relationship between sensitivity and specificity of palladin as a diagnostic test for the presence of hepatic metastases is shown in Figure 4. Diagnostic tests that discriminate well have an area under the ROC curve that approaches 1, whereas those tests that perform poorly have a curve that falls close to the diagonal dashed line and an area under the curve closer to 0.5. The area under the ROC curve of 0.8780 indicated that palladin expression is a good discriminatory test for the presence of hepatic metastases. The ROC analysis was used to choose the Allred score cut-off point for palladin expression that would achieve maximum sensitivity in identifying hepatic metastases without compromising specificity (increasing false-positive rate). The sensitivity of an Allred score of 3 or greater for predicting hepatic metastases was 100%, specificity was 63% and overall predictive accuracy was 76%. Choice of a lower cut-off point would not increase sensitivity any further, but would reduce specificity. Choosing a higher Allred score cut-point would lead to a loss of sensitivity from 100% to 79% that would exceed the gain in specificity from 63% to 75%. Choosing an Allred score cut-off point of 3 or greater correctly classified the pancreatic endocrine neoplasms as having hepatic metastases or not (predictive accuracy) in 76% of the cases.

Table IV. *Pathologic staging of pancreatic endocrine tumors using the TNM-based criteria established by AJCC Staging Manual 2010 (Ref. 3).*

T-stage	N-stage	M-stage
T1 – 7 (18%)	NX – 11 (29%)	M0 – 24 (63%)
T2 – 19 (50%)	N0 – 16 (42%)	M1 – 14 (29%)
T3 – 11 (29%)	N1 – 11 (29%)	
T4 – 1 (3%)		

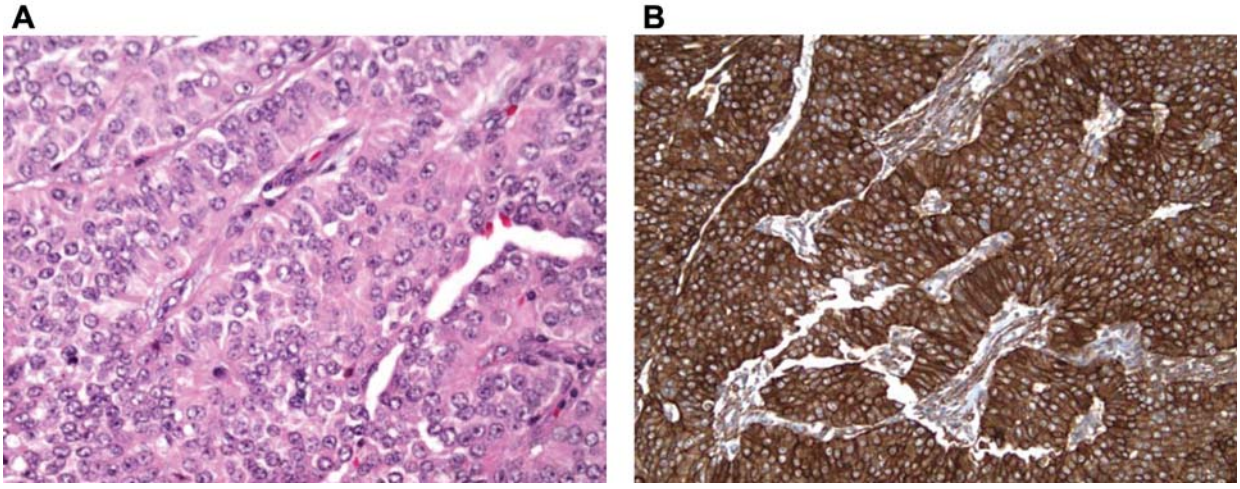

Figure 3. *A primary, well-differentiated pancreatic endocrine carcinoma that had clinical evidence of synchronous liver metastasis (MP-PECA) at the time of resection. A: Hematoxylin and eosin stain (original magnification ×200). B: The same tumor exhibiting diffuse and strong cytoplasmic expression of palladin (original magnification ×200).*

Prediction of liver metastases based on pathological tumor characteristics. None of the pathological criteria regarded as markers demonstrated statistically significant association with hepatic metastases (Table III). Only high palladin expression (Allred score ≥3) was strongly associated with hepatic metastases ($p<0.0001$). Tumor necrosis was uncommon, as it was seen in only 6 out of the 38 tumors (16%). And of these cases, 4 were already metastatic to the liver, while 2 were still clinically localized Tables I and II.

Discussion

The immunohistochemical expression of palladin protein in primary pancreatic endocrine tumors may represent a potential predictor of hepatic metastases. In contrast, the other pathologic tumor characteristics analyzed showed no statistical significance with the presence of liver metastases (Table III). These findings provide evidence that over-expression of palladin in pancreatic neuroendocrine tumors may represent a novel prognostic marker of liver metastasis.

This was in line with prior reports relating palladin to the invasive and metastatic capability of cancer cells (14, 15). Within pancreatic and colorectal malignancies, palladin is included in a cluster of invasion-specific genes (14). The upregulation of the palladin gene as a part of the molecular signature of invasion in breast cancer cells (15). Taken together, these studies suggest that palladin over-expression may contribute to the development of tumor metastases.

In the present study, palladin expression was found in the majority of primary pancreatic endocrine neoplasms. Although in other tumor types, palladin expression has been reported to be less frequent, for example, palladin expression was reported in only 12.4% of 177 pancreatic adenocarcinomas cases (16). In the present study, palladin expression in the peri- and in the intra-tumoral stroma has also been reported by others (16). The stromal localization of palladin may reflect the association of this protein with F-actin filaments and its modulation of tumor cell motility and stromal remodeling (4), which warrants further investigation. Since palladin is also known to bind other proteins such as esp8, ezerin, profiling and vasp (4, 5, 7), additional studies focused on these palladin-related proteins may define other prognostic biomarkers for pancreatic endocrine tumors.

The present finding of palladin expression as a predictor for metastatic potential of PNETs was in agreement with a recent

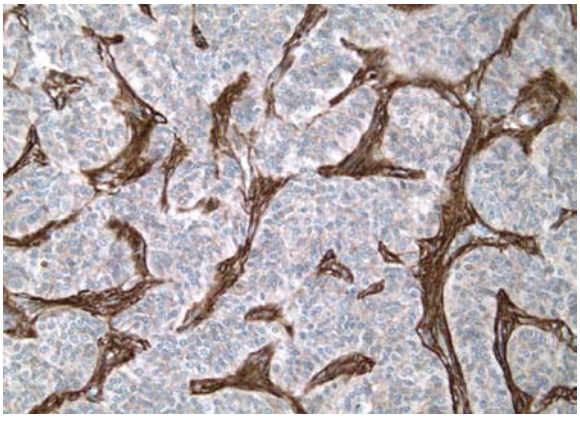

Figure 4. *A primary, well-differentiated pancreatic endocrine tumor that had no clinical evidence of liver metastasis (CLP-PET) at the time of resection of the pancreatic primary showing no palladin expression in the tumor cells. However, the intratumoral stroma is palladin-positive (Palladin immunostain, original magnification ×200).*

study showing increased palladin expression in metastatic breast carcinomas as compared to their primary tumors (11). The same investigators reported that highly invasive breast cancer cell lines expressed significantly higher levels of palladin than non-invasive breast cancer cell lines (15) and that siRNA-mediated knockdown decreased tumor cells' migratory and invasive capabilities.

The overexpression of palladin protein seems to contribute to the abnormalities in the cytoskeleton of the pancreatic endocrine tumor cells with consequent increase in their ability to migrate into surrounding tissues. Additionally, palladin expression may be an early event in the carcinogenesis of PETs due to the fact that histologically normal pancreatic islets from the MP-PECAs in our study showed an increased expression of palladin. The association of palladin with MP-PECA may identify patients with a propensity to develop synchronous or metachronous liver metastases, and thus with poorer prognosis.

Since the HNPIs sampled from areas adjacent to the MP-PECAs exhibited higher palladin expression as compared to those from the CLP-PETs, palladin up-regulation may represent a molecular change driving neuroendocrine tumor cells to liver metastasis. Additional retrospective and prospective studies on a larger selection of clinically localized and metastatic pancreatic endocrine neoplasms are needed to further explore the clinical utility of palladin as a marker of liver metastases.

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Conflict of Intrest

The Authors have no conflict of interest to disclose.

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