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Expression of Estrogen-Induced Genes and Estrogen Receptor Beta in Pancreatic Neuroendocrine Tumors: Implications for Targeted Therapy

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Abstract

Objective—The indolent nature and expression of progesterone receptor (PR), a well-known estrogen-induced gene, in a subset of pancreatic neuroendocrine tumors (PanNETs), raise the possibility of hormonal regulation in these tumors.

Methods—Immunohistochemical expression of estrogen receptors (ER) α and β and mRNA expression of estrogen-induced genes (*PR*, *EIG121*, *IGF-1*, *IGF-1R*, *sFRP1*, and *sFRP4*) by qRT-PCR were examined in 131 WHO G1-G2 PanNETs and correlated expression with clinicopathologic features.

Results—39 PanNETs (30%) showed high positive ER β staining and 87 cases (66%) had low positive ER β staining; only 5 cases (4%) had no nuclear staining. PanNETs with small size ($P=.02$), low WHO grade ($P=.02$) and low AJCC stage ($P=.006$) more frequently showed high positive ER β staining. Among estrogen-induced genes studied, PanNETs had significantly higher expression of *PR*, *EIG121*, *IGF-1*, *sFRP1*, and *sFRP4* compared to normal pancreas, independent of age or gender. High positive ER β staining was associated with increased expression of *PR* ($P < .001$) and *EIG121* ($P=.02$).

Conclusions—Our study showed that PanNETs with favorable prognostic features have higher ER β expression, which is associated with upregulated *PR* and *EIG121* mRNA expression. Estrogen regulation in PanNETs could potentially help in risk stratification and provide a rational target for novel treatment strategies.

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Keywords

Pancreatic neuroendocrine tumor; ER β ; estrogen-induced genes

INTRODUCTION

Pancreatic neuroendocrine neoplasms are rare, representing approximately 3% of primary pancreatic neoplasms¹, although their incidence has risen sharply in the United States, increasing more than 100% over the past three decades². They have a highly variable clinical behavior, ranging from indolent to highly aggressive. Up to 61% of patients present with distant metastases and only 26% of whom undergo surgical resection³. The poor survival in some patients is due, in part, to the limited treatment options for these patients⁴. Platinum-based chemotherapy with radiation and surgery may be a treatment option for patients with high-grade neuroendocrine carcinoma (HGNEC), however, studies suggest that these tumors are clinically and molecularly more similar to small cell carcinomas of other sites and distinct from well-differentiated [low (G1) and intermediate grade (G2)]⁵ pancreatic neuroendocrine tumors (PanNETs)^{6–8}. For patients with PanNETs, surgery remains the primary treatment option. Recently, two new targeted therapies have been recently approved by the U.S. Food and Drug Administration for patients with advanced PanNETs. Sunitinib (vascular endothelial growth factor receptor-tyrosine kinase inhibitor) and everolimus (mammalian target of rapamycin inhibitor) have been shown in phase III trials to improve progression-free survival compared to placebo⁴. Despite these advances, treatment of patients with advanced PanNET remains unclear⁹. One important challenge in therapy development for PanNETs is that the mechanisms of tumorigenesis are not clear.

The indolent nature and the expression of progesterone receptor (PR), a well-known estrogen-induced gene, in some PanNETs raise the question of possible hormonal regulation in these neoplasms. Substantial subsets of prostate, breast, and endometrial carcinoma are similarly indolent; these subsets are characterized by hormonal dependence. For prostatic carcinoma, it is well-known that metastasis and aggressive clinical course are tightly linked to androgen independence. Androgen-independent tumors typically have high-grade neuroendocrine features and patients survive < 1 year¹⁰. It is well-documented that in carcinomas of the breast and endometrium, estrogen regulation has played a pivotal role in tumor subclassification, risk assessment and treatment strategies. Estrogen receptor (ER) status has remained one of the most important molecular markers in the management of patients with breast cancer, as it provides both prognostic and predictive value¹¹. In endometrial carcinoma, classification of tumors into type I and type II categories has been traditionally based, in part, on ER status, namely, most type I tumors are ER positive while most type II tumors are ER negative¹². Moreover, quantitative assessment of expression of genes induced by estrogen, including *IGF-1*, *IGF-1R*, *RALDH2*, *sFRP1*, *sFRP4*, and *EIG121*^{13–15}, has been shown to provide important prognostic data for endometrial carcinoma¹⁴. Specifically, high expression of estrogen-induced genes in endometrial carcinoma is associated with endometrioid histotype and significantly prolonged recurrence-free survival¹⁴.

The role of estrogen regulation in PanNETs is unclear, and studies on ER expression are conflicting with reported rates of 0–13% ER alpha positivity by immunohistochemistry^{16, 17} and up to 40% positivity in insulinomas¹⁸. However, previous studies have shown that PR, is important in islet cell function^{19–22} and has prognostic significance in PanNETs^{12, 17, 23}. Loss of PR immunohistochemical expression is associated with presence of metastasis, lymphovascular invasion and involvement of adjacent organs¹⁷. Moreover, two previous reports have shown that loss of PR expression correlated with shorter disease-free¹² and overall²³ survival by univariate analysis.

Given these previous findings, we hypothesized that low and intermediate grade PanNETs would express the hormone receptors ER α and/or ER β and, thus, estrogen-induced genes. Results from this study can potentially provide insight into hormonal regulation and novel hormonal therapeutic targets in PanNETs.

MATERIALS AND METHODS

Patients and tissue samples

Formalin-fixed paraffin-embedded PanNET tumor sections from routinely processed partial pancreatectomy specimens (including pancreaticoduodenectomy, distal pancreatectomy and enucleation) and/or resected metastases were retrieved from the Surgical Pathology files at The University of Texas M. D. Anderson Cancer Center, Houston, TX, between 2000 – 2010. A total of 131 PanNETs, low grade (G1) and intermediate grade (G2) according to the World Health Organization (WHO) criteria⁵, were included. High-grade pancreatic neuroendocrine carcinomas were specifically excluded, as these tumors have different clinical behavior and molecular profile from G1 and G2 PanNETs⁷. Clinical data were extracted from patient medical records. Functionality was defined as documented increase in blood hormone levels and concomitant constellation of typical signs and symptoms, as defined by the WHO⁵. Hematoxylin and eosin (H&E)-stained slides 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 American Joint Committee on Cancer (AJCC) Staging Manual, 7th edition criteria²⁴. The study was approved by the Institutional Review Board (IRB) of The University of Texas M. D. Anderson Cancer Center.

Immunohistochemistry

Immunohistochemistry were initially performed on tissue microarrays (TMA) of 80 PanNETs and validated using 5 μ m-thick formalin-fixed (10% buffered formalin), paraffin-embedded whole tissue sections of 131 PanNETs. Standard avidin-biotin complex (ABC) technique was employed using antibodies to Ki-67 (clone MIB-1, 1:100; DAKO, Carpinteria, CA), ER α (clone 6F11, 1:35; Novocastra), and ER β (clone EMR02, 1:100, Leica microsystems, Buffalo Grove, IL). Quantification of the proliferative index by Ki-67 immunohistochemistry was performed using computer-assisted image analysis with Aperio ImageScope Version 11.2.0.780 (copyright © Aperio Technologies, Inc. 2003 – 2012) and counting at least 2,000 tumor nuclei in the areas with the highest percentage of nuclear staining (“hotspots”), as per the 2010 WHO guidelines⁵. PanNETs were categorized as low

grade (G1, 0–2.9% Ki-67 labeling) or intermediate grade (G2, 3–20% Ki-67 labeling). High-grade pancreatic neuroendocrine carcinomas, those with greater than 20% Ki-67 labeling, were excluded. Nuclear staining for ER α in greater than or equal to 1% of the whole tumor section was considered positive, while those cases with less than 1% were considered negative, a cut-off used in breast cancer^{25, 26}. Nuclear staining for ER β was evaluated using computer-assisted image analysis with Aperio ImageScope Version 11.2.0.780 (copyright © Aperio Technologies, Inc. 2003 – 2012) and evaluating a representative area, attempting to encompass most of the tumor section. ER β was scored using the Allred scoring system used in breast carcinoma^{26, 27}. Briefly, the proportion of positively stained tumor nuclei (0, none; 1, < 1/100; 2, 1/100 to 1/10; 3, 1/10 to 1/3; 4, 1/3 to 2/3; and 5, > 2/3) and intensity of the majority of tumor cells (0, none; 1, weak; 2, intermediate; and 3, strong) were added to obtain a total score, ranging from 0 to 8. ER β immunohistochemical staining was then categorized as high positive (Allred scores = 7–8), low positive (Allred scores = 3–6) and negative (Allred score = 0).

RNA isolation, reverse transcription and real-time quantitative PCR

H&E stained sections of PanNET from formalin-fixed, paraffin-embedded tissue blocks were microscopically analyzed by a gastrointestinal pathologist (JSE) to confirm the presence of low-intermediate grade tumor. Forty-one cases had sufficient normal pancreatic tissue that was used as control. Briefly, twenty 10- μ m sections were cut from each paraffin block and then deparaffinized in xylene. T&C Lysis solution (Master Pure RNA Purification Kit, catalogue number MCR 85102, Madison, WI) with proteinase K was used for tissue digestion. RNA was extracted using the MasterPure Reagent Kit (Epicenter, Madison, WI). The real-time quantitative RT-PCR (qRT-PCR) assays were performed using the 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Probe-based real-time quantitative assays were initially developed using Primer Express software (Applied Biosystems) based on sequences from Genbank. The sequences for qRT-PCR analyses of the estrogen-induced genes *PR*, *EIG121*, *IGF-I*, *IGF-IR*, *sFRP1*, and *sFRP4* are listed in Table 1. Each qRT-PCR experiment was performed in duplicate using assay-specific sDNAs (synthetic amplicon oligonucleotides, Biosource, Camarillo, CA) serially diluted in 10-fold decrements to obtain a standard curve covering a 5-log range in template concentration. A linear relationship between the threshold cycle (Ct) and the log of the starting sDNA copy number was established (correlation coefficient > 0.99) and used to construct a standard curve. The copy number for each transcript assayed was interpolated from the standard curve by the ABI SDS software. The final transcript values were normalized to 18S rRNA and were presented as ratios of gene transcript/molecules of 18S rRNA. We used 18S rRNA as a normalizer, because levels of 18S rRNA do not fluctuate significantly over time or with exposure to hormones.

Statistical Methods

Associations between ER β staining category and clinicopathologic categorical data were determined using Fisher's exact test. Associations between estrogen-induced gene mRNA expression and continuous variables were assessed using Spearman's correlation coefficient (ρ), and categorical variables using either Wilcoxon rank sum test (those with two groups) or Kruskal-Wallis test (those with three groups). All statistical analyses were performed using

IBM SPSS Statistics version 21 for Windows (Copyright © 1989, 2012 by IBM Corporation).

RESULTS

Clinicopathologic characteristics

Patients' clinicopathologic characteristics are summarized in Table 2. Mean age was 56 years (range: 18 – 79 years). The female to male ratio was 1:1.6. Twenty females were of pre-menopausal age (< 50 years old) while 30 females were of post-menopausal age (≥ 50 years old). Eleven patients (8%) had associated multiple endocrine neoplasia, type 1 (MEN1). Twenty-two (17%) PanNETs were functional, including 14 insulinomas, 5 gastrinomas and 3 glucagonomas. Mean tumor size was 4.5 cm (range: 0.7 – 20 cm). According to the WHO classification standards⁵, 55% (72 cases) were G1 PanNETs and 45% (59 cases) were G2 PanNETs. Based on the AJCC (7th edition) staging manual, 35 patients (27%) had stage I disease, 59 patients (45%) had stage II disease and 37 patients (28%) had stage IV disease. No patient had stage III disease, as those patients would not be surgical candidates. Liver was the most common site of distant metastasis at presentation (29 patients, 78%).

ERα and ERβ immunohistochemistry

Immunohistochemistry for ERα was first assessed in a subset of PanNETs using TMAs (n=80). None of these had any detectable ER nuclear staining, so additional studies with ERα were not pursued. ERβ immunohistochemical staining was present in the majority of PanNETs. We, next, investigated ERβ protein expression by immunohistochemistry in 131 PanNETs using whole tissue sections. The majority of PanNETs (96%) exhibited nuclear ERβ. In these tumors, ERβ immunostaining was typically stronger than the adjacent normal pancreatic acini, ducts and islets of Langerhans (Figures 1A-B). By Allred scoring, ERβ staining was as follows: Allred score = 8 (18 cases), 7 (21 cases), 6 (50 cases), 5 (23 cases), 4 (10 cases), 3 (4 cases), and 0 (5 cases).

Among 131 PanNETs, 39 (30%) showed high levels of ERβ staining (high positive, Figure 1A-B). Low levels of ERβ staining were seen in 87 (66%) cases (low positive). Five cases (4%) had no nuclear ERβ staining (negative, Figures 1C-D). Correlation with ERβ immunohistochemistry and clinicopathologic features are summarized in Table 3. ERβ staining correlated with tumor size ($P=.02$), WHO grade ($P=.02$), and AJCC stage ($P=.006$). No significant correlation between ERβ immunohistochemical staining and age, including reproductive age, gender, functional tumors, and MEN1 syndrome, were seen.

mRNA expression of estrogen-induced genes

Given that the majority of PanNETs had immunohistochemical expression of ERβ, we next wanted to determine whether PanNETs expressed genes known to be induced by estrogen. PanNETs showed increased mRNA expression of *PR*, *EIG121*, *IGF-1*, *sFRP1* and *sFRP4*, compared to normal pancreas. Expression of these genes was independent of age, including reproductive age, and gender (Table 4). Of note, there was a 10-fold increase in *EIG121*

mRNA expression compared to other estrogen-induced genes, suggesting that *EIG121* may be a dominant estrogen regulated gene in PanNETs.

We, then, analyzed the association between ER β immunohistochemical staining and expression of the estrogen-induced genes (Table 4). High ER β expression was associated with increased expression of *PR* and *EIG121* mRNA expression. These results suggest that of the genes examined in this study, expression of *PR* and *EIG121* may be the most tightly linked to ER β protein expression. Among those associated with ER β immunohistochemical staining, increased *PR* mRNA expression was seen in tumors with low grade ($P=.03$), and low stage ($P=.003$), and increased *EIG121* mRNA expression was seen in low stage ($P=.02$), but did not correlate with WHO grade. Correlation between mRNA expression and clinicopathologic characteristics are summarized in Table 5.

DISCUSSION

In this study of 131 PanNETs, we demonstrated that most PanNETs express the hormone receptor ER β , and that the ER β Allred score significantly correlated with expression of some estrogen-induced genes, suggesting a role of ER β in their regulation. Our study also showed that high levels of ER β immunohistochemical staining and high expression of some estrogen-induced genes were significantly associated with PanNETs with small size, low WHO grade and low AJCC stage, suggesting that this “hormonal” subset of PanNETs is associated with better prognostic features. The novelty of this study is that it demonstrates the possible involvement of estrogen regulation in PanNET tumorigenesis, thereby providing rationale for molecular subclassification and novel hormonal therapeutic strategies. For an indolent malignancy such as PanNET, hormonal approaches may be an attractive therapeutic option for patients.

ER α and ER β are both members of the nuclear steroid receptor superfamily of transcription. They are present on distinct chromosomes; ER α is located on chromosome 6 (6q25)²⁸ while ER β is located on chromosome 14 (14q22-24)²⁹. Both have a highly conserved DNA binding domain region which allows them to bind to similar target sites, but the activation function domain (AF-1, involved in protein-protein interaction³⁰ and transcriptional activation³¹), hinge region (nuclear localization signal), ligand binding domain, coregulator binding surface, dimerization domain, and activation function 2 domain (AF-2, involved in hormone-dependent activation³²) are not well conserved (30 – 53% homology between the two receptors)³³. While both exhibit similar affinities for estradiol, they show different affinities for a wide variety of other compounds including anti-estrogens and phytoestrogens³⁴. Although they have been shown to have overlapping organ distribution, they also localize to distinct tissue and/or cell subtype in the same tissue or exhibit differing levels of expression in the same tissue³⁴. These differences may account for the differences in their tissue specific biologic action.

The previous studies on ER α immunohistochemical expression in PanNETs showed conflicting results. Alabraba, et al found ER (alpha) positivity in 10 of 25 insulinomas (40%)¹⁸, Arnason, et al. found only weak and focal staining in 5 of 40 tumors (12.5 %) with no tumors exhibiting strong nuclear staining in their study¹² while Viale, et al. (n=96)¹⁷

found no ER staining in the tumors analyzed. Similar to Viale, et al., we found no ER α staining in the 80 PanNETs included in our TMAs, which included 5 insulinomas.

On the other hand, ER β immunohistochemistry has been reported in insulinomas, although studies on other types of PanNETs, non-functional tumors and non-insulinomas, are lacking. In the study by Alabraba, et al. analyzing 25 insulinomas, they also found strong, diffuse (greater than 50% of tumor cells) immunohistochemical staining in 20 of 25 and weak, focal (less than 50% of tumor cells) staining in one other case, totaling 21 of 25 (84%) PanNETs with positive immunohistochemical staining for ER β ¹⁸. In our study, we showed that strong diffuse nuclear ER β immunohistochemical staining was present in normal pancreatic acinar cells, ductal epithelium and islets of Langerhans (Figure 1) and 30% of PanNETs, low positivity was seen in 66% of PanNETs while only 4% of PanNETs had no nuclear staining for ER β . Our results are compatible with the high frequency of ER β staining previously reported in insulinomas¹⁸.

The physiologic role of ER β in the pancreas is unclear. Previous studies suggest a role for ER β in β -cell function. *In vitro* studies have shown that 17 β -estradiol regulates glucose-stimulated insulin secretion by inhibiting ATP-sensitive potassium channels through ER β in mouse pancreatic β -cells³⁵, as well as in human islets of Langerhan cells³⁶. In addition, the selective ER β agonist WAY200070 restores β -cell mass by increased proliferation in streptozotocin-nicotinamide-induced diabetic mice³⁶. The mechanism through which ER β increases β -cell proliferation remains unknown. Based on our results, we hypothesize that dysregulation of this ER β signaling contributes to the development of PanNETs.

It is well established that increased signaling through hormone receptors plays an important role in the development of large subsets of breast, endometrial and prostate cancers. Cancers with intact expression of ER α and AR are typically slow-growing and confer a better prognosis than tumors that are negative for hormone receptor expression. ER β has similar properties in cancers. *In vitro* studies of ovarian cancer cell lines have shown that ER β inhibited cell proliferation by decreasing the proportion of cells in S phase and increasing the proportion of cells in G2/M phase by inhibiting Akt phosphorylation thereby reducing cyclin D1 expression, and in turn downregulating phosphorylation of Rb³⁷. In breast cancer cell lines, ER β induced G2 cell cycle arrest by downregulating cyclin D1, cyclin A and c-myc expression thereby upregulating expression of p21 and p27, in a ligand-independent mechanism³⁸. Moreover, studies on patients with breast carcinoma have shown that ER β immunohistochemical expression correlated with negative axillary node status ($P < .0001$)³⁹ and low grade ($P=.0003$)³⁹, was an independent predictor of longer disease-free survival (HR=0.264, 95% CI 0.12 – 0.58, $P=.001$)⁴⁰ and overall survival (HR=0.384, 95% CI 0.18 – 0.80, $P=.01$)⁴⁰ by multivariate analysis. Among PanNETs in our study, we found that high immunohistochemical positivity for ER β significantly correlated with small size ($P=.01$), low WHO grade (G1, $P=.02$) and low AJCC stage (stage I-IIb, $P=.006$).

Among the estrogen-induced genes evaluated, we found that *PR* and *EIG121* mRNA expression were significantly correlated with ER β immunohistochemical staining in PanNETs, suggesting a possible role for ER β in their regulation. The remaining genes in the panel utilized for this study, while regulated by estrogen in the female endometrium, may be

regulated by other factors in addition to estrogen in the pancreas. The utility of PR immunohistochemistry as a prognostic factor has been shown in previous studies. Viale, et al. 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 ($P=0.0003$)¹⁷. Pelosi, et al. analyzed 54 PanNETs and showed that absent PR staining correlated with shorter survival ($P=0.013$) by univariate analysis²³. Recently, we found that patients with PR-negative PanNETs had a significantly worse overall survival, independent of AJCC stage (HR=3.1, CI=1.4-7.1, $P=0.007$)⁴¹. Our study is the first to evaluate the prognostic significance of *PR* mRNA expression in PanNETs. In agreement with immunohistochemical studies, decreased *PR* mRNA expression was significantly associated with WHO intermediate grade and AJCC stage IV disease.

EIG121 upregulation has been shown in type I endometrial carcinomas, tumors associated with estrogen regulation and generally favorable prognosis. Deng, et al. analyzed *EIG121* mRNA expression in endometrium of premenopausal and postmenopausal women and found a 1.9-fold increase in proliferative (estrogen-dominated) phase compared to secretory phase and a 3.8- and > 21-fold increase in endometrial hyperplasia and grade 1 endometrioid carcinoma, respectively, compared to normal endometrium¹⁵. In their study, they also found that *EIG121* was significantly down-regulated in type II endometrial carcinomas, which are typically not associated with estrogen exposure and, present with advanced stage and confer worse patient prognosis. Our study is the first to investigate the expression of *EIG121* in PanNETs. Similar to other carcinomas, we found upregulation of *EIG121* mRNA expression in tumors with low AJCC stage. We have previously shown that *EIG121* regulates autophagy and promotes cell survival under stress⁴², however, the function of high *EIG121* in PanNETs is not entirely clear.

The finding that PanNETs with small size, low WHO grade and/or low AJCC stage disease showed high immunohistochemical positivity for ER β and upregulation of *PR* and *EIG121* mRNA expression suggest that a subset of PanNETs, namely those with indolent disease, may be molecularly distinct from intermediate grade tumors and those with advanced disease. As we have learned from breast and uterine carcinomas, the heterogeneity of patient outcome may be influenced, in part, by hormone receptor status. Molecular classification of breast carcinoma into ER+/HER2-, ER+/HER2+, and ER-/HER2-, described by Kapp, et al.⁴³ has significantly helped in risk assessment and guiding optimal treatment of patients. Similarly, classification of endometrial carcinomas into type I and type II has been traditionally based on tumor response to estrogen and ER status¹⁶. Molecular subclassification of PanNETs based on estrogen regulation may help in risk stratification. Our results demonstrate that routinely acquired formalin-fixed paraffin embedded patient material may be used effectively for this purpose.

To date, the primary treatment of patients with PanNETs remains surgery. Two new targeted therapies have been recently approved by the U.S. Food and Drug Administration for patients with advanced PanNETs. Sunitinib (vascular endothelial growth factor receptor-tyrosine kinase inhibitor) and everolimus (mammalian target of rapamycin inhibitor) have been shown in phase III trials to improve progression-free survival compared to placebo⁴. Despite these advances, treatment of patients with advanced PanNET remains sub-optimal⁹.

ER β has been identified as a potential molecular target in breast, ovarian, prostate and colon cancer chemoprevention by the American Association for Cancer Research task Force Report⁴⁴. Evidence presented here suggests that targeting ER β in a subset of patients with PanNETs may provide another treatment avenue.

In summary, we found that a subset of PanNETs, those with indolent behavior, have upregulated *PR* and *EIG121* mRNA expression, which may be regulated by ER β . Thus, estrogen regulation in PanNETs may uncover the underlying mechanisms driving outcome heterogeneity and could potentially provide a rational target for novel treatment strategies in patients with PanNETs.

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REFERENCES

1. Fesinmeyer MD, Austin MA, Li CI, et al. Differences in survival by histologic type of pancreatic cancer. *Cancer Epidemiol Biomarkers Prev.* 2005; 14:1766–1773. [PubMed: 16030115]
2. Yao JC, Hassan M, Phan A, et al. One hundred years after "carcinoid": epidemiology of and prognostic factors for neuroendocrine tumors in 35,825 cases in the United States. *J Clin Oncol.* 2008; 26:3063–3072. [PubMed: 18565894]
3. Bilimoria KY, Bentrem DJ, Merkow RP, et al. Application of the pancreatic adenocarcinoma staging system to pancreatic neuroendocrine tumors. *J Am Coll Surg.* 2007; 205:558–563. [PubMed: 17903729]
4. Yao JC, Lagunes DR, Kulke MH. Targeted Therapies in Neuroendocrine Tumors (NET): Clinical Trial Challenges and Lessons Learned. *Oncologist.* 2013; 18:525–532. [PubMed: 23615698]
5. Klimstra, DS.; Arnold, R.; Capella, C., et al. Nomenclature and classification of neuroendocrine neoplasms of the digestive system. In: Bosman, FT.; Carneiro, F.; Hruban, RH., et al., editors. *WHO classification of tumours of the digestive system.* 4th ed.. Lyon: International Agency for Research on Cancer; 2010. p. 322-326.
6. Smith J, Reidy-Lagunes D. The management of extrapulmonary poorly differentiated (high-grade) neuroendocrine carcinomas. *Semin Oncol.* 2013; 40:100–108. [PubMed: 23391117]
7. Yachida S, Vakiani E, White CM, et al. Small cell and large cell neuroendocrine carcinomas of the pancreas are genetically similar and distinct from well-differentiated pancreatic neuroendocrine tumors. *Am J Surg Pathol.* 2012; 36:173–184. [PubMed: 22251937]
8. Rindi G, Bordi C. Highlights of the biology of endocrine tumours of the gut and pancreas. *Endocr Relat Cancer.* 2003; 10:427–436. [PubMed: 14713255]
9. Kunz PL, Reidy-Lagunes D, Anthony LB, et al. Consensus Guidelines for the Management and Treatment of Neuroendocrine Tumors. *Pancreas.* 2013; 42:557–577. [PubMed: 23591432]
10. Tetu B, Ro JY, Ayala AG, et al. Small cell carcinoma of the prostate. Part I. A clinicopathologic study of 20 cases. *Cancer.* 1987; 59:1803–1809. [PubMed: 3030528]
11. Allred DC, Carlson RW, Berry DA, et al. NCCN Task Force Report: Estrogen Receptor and Progesterone Receptor Testing in Breast Cancer by Immunohistochemistry. *J Natl Compr Canc Netw.* 2009; 7(Suppl 6):S1–S21. quiz S22-3. [PubMed: 19755043]
12. Arnason T, Sapp HL, Barnes PJ, et al. Immunohistochemical expression and prognostic value of ER, PR and HER2/neu in pancreatic and small intestinal neuroendocrine tumors. *Neuroendocrinology.* 2011; 93:249–258. [PubMed: 21487213]

13. Deng L, Shipley GL, Loose-Mitchell DS, et al. Coordinate regulation of the production and signaling of retinoic acid by estrogen in the human endometrium. *J Clin Endocrinol Metab.* 2003; 88:2157–2163. [PubMed: 12727970]
14. Westin SN, Broaddus RR, Deng L, et al. Molecular clustering of endometrial carcinoma based on estrogen-induced gene expression. *Cancer Biol Ther.* 2009; 8:2126–2135. [PubMed: 19755863]
15. Deng L, Broaddus RR, McCampbell A, et al. Identification of a novel estrogen-regulated gene, EIG121, induced by hormone replacement therapy and differentially expressed in type I and type II endometrial cancer. *Clin Cancer Res.* 2005; 11:8258–8264. [PubMed: 16322283]
16. Lax SF, Kurman RJ. A dualistic model for endometrial carcinogenesis based on immunohistochemical and molecular genetic analyses. *Verh Dtsch Ges Pathol.* 1997; 81:228–232. [PubMed: 9474874]
17. Viale G, Doglioni C, Gambacorta M, et al. Progesterone receptor immunoreactivity in pancreatic endocrine tumors. An immunocytochemical study of 156 neuroendocrine tumors of the pancreas, gastrointestinal and respiratory tracts, and skin. *Cancer.* 1992; 70:2268–2277. [PubMed: 1356613]
18. Alabraba EB, Taniere P, Reynolds GM, et al. Expression and functional consequences of oestrogen and progesterone receptors in human insulinomas. *Endocr Relat Cancer.* 2007; 14:1081–1088. [PubMed: 18045959]
19. Nieuwenhuizen AG, Schuiling GA, Hilbrands LG, et al. Proliferation of pancreatic islet-cells in cyclic and pregnant rats after treatment with progesterone. *Horm Metab Res.* 1998; 30:649–655. [PubMed: 9918380]
20. Magnaterra R, Porzio O, Piemonte F, et al. The effects of pregnancy steroids on adaptation of beta cells to pregnancy involve the pancreatic glucose sensor glucokinase. *J Endocrinol.* 1997; 155:247–253. [PubMed: 9415059]
21. Shao J, Qiao L, Friedman JE. Prolactin, progesterone, and dexamethasone coordinately and adversely regulate glucokinase and cAMP/PDE cascades in MIN6 beta-cells. *Am J Physiol Endocrinol Metab.* 2004; 286:E304–E310. [PubMed: 14559722]
22. Picard F, Wanatabe M, Schoonjans K, et al. Progesterone receptor knockout mice have an improved glucose homeostasis secondary to beta -cell proliferation. *Proc Natl Acad Sci U S A.* 2002; 99:15644–15648. [PubMed: 12438645]
23. Pelosi G, Bresaola E, Bogina G, et al. Endocrine tumors of the pancreas: Ki-67 immunoreactivity on paraffin sections is an independent predictor for malignancy: a comparative study with proliferating-cell nuclear antigen and progesterone receptor protein immunostaining, mitotic index, and other clinicopathologic variables. *Hum Pathol.* 1996; 27:1124–1134. [PubMed: 8912819]
24. Edge SB.; Byrd DR.; Compton CC., et al., editors. *AJCC cancer staging manual.* 7th ed.. New York: Springer; 2010. p. 241-246.
25. Hammond ME, Hayes DF, Wolff AC. Clinical Notice for American Society of Clinical Oncology-College of American Pathologists guideline recommendations on ER/PgR and HER2 testing in breast cancer. *J Clin Oncol.* 2011; 29:e458. [PubMed: 21502545]
26. Harvey JM, Clark GM, Osborne CK, et al. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. *J Clin Oncol.* 1999; 17:1474–1481. [PubMed: 10334533]
27. Fuqua SA, Schiff R, Parra I, et al. Estrogen receptor beta protein in human breast cancer: correlation with clinical tumor parameters. *Cancer Res.* 2003; 63:2434–2439. [PubMed: 12750263]
28. Gosden JR, Middleton PG, Rout D. Localization of the human oestrogen receptor gene to chromosome 6q24----q27 by in situ hybridization. *Cytogenet Cell Genet.* 1986; 43:218–220. [PubMed: 3802924]
29. Enmark E, Peltö-Huikko M, Grandien K, et al. Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. *J Clin Endocrinol Metab.* 1997; 82:4258–4265. [PubMed: 9398750]
30. Onate SA, Boonyaratankornkit V, Spencer TE, et al. The steroid receptor coactivator-1 contains multiple receptor interacting and activation domains that cooperatively enhance the activation

- function 1 (AF1) and AF2 domains of steroid receptors. *J Biol Chem.* 1998; 273:12101–12108. [PubMed: 9575154]
31. Bardin A, Hoffmann P, Boulle N, et al. Involvement of estrogen receptor beta in ovarian carcinogenesis. *Cancer Res.* 2004; 64:5861–5869. [PubMed: 15313930]
32. Tremblay GB, Tremblay A, Copeland NG, et al. Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta. *Mol Endocrinol.* 1997; 11:353–365. [PubMed: 9058381]
33. Pearce ST, Jordan VC. The biological role of estrogen receptors alpha and beta in cancer. *Crit Rev Oncol Hematol.* 2004; 50:3–22. [PubMed: 15094156]
34. Gallo D, De Stefano I, Grazia Prisco M, et al. Estrogen receptor beta in cancer: an attractive target for therapy. *Curr Pharm Des.* 2012; 18:2734–2757. [PubMed: 22390760]
35. Soriano S, Roperio AB, Alonso-Magdalena P, et al. Rapid regulation of K(ATP) channel activity by 17{beta}-estradiol in pancreatic {beta}-cells involves the estrogen receptor {beta} and the atrial natriuretic peptide receptor. *Mol Endocrinol.* 2009; 23:1973–1982. [PubMed: 19855088]
36. Alonso-Magdalena P, Roperio AB, Garcia-Arevalo M, et al. Antidiabetic actions of an estrogen receptor beta selective agonist. *Diabetes.* 2013; 62:2015–2025. [PubMed: 23349481]
37. Bossard C, Busson M, Vindrieux D, et al. Potential role of estrogen receptor beta as a tumor suppressor of epithelial ovarian cancer. *PLoS One.* 2012; 7:e44787. [PubMed: 22970307]
38. Paruthiyil S, Parmar H, Kerekatte V, et al. Estrogen receptor beta inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle arrest. *Cancer Res.* 2004; 64:423–428. [PubMed: 14729654]
39. Jarvinen TA, Peltto-Huikko M, Holli K, et al. Estrogen receptor beta is coexpressed with ERalpha and PR and associated with nodal status, grade, and proliferation rate in breast cancer. *Am J Pathol.* 2000; 156:29–35. [PubMed: 10623650]
40. Nakopoulou L, Lazaris AC, Panayotopoulou EG, et al. The favourable prognostic value of oestrogen receptor beta immunohistochemical expression in breast cancer. *J Clin Pathol.* 2004; 57:523–528. [PubMed: 15113861]
41. Estrella J, Broaddus R, Mathews A, et al. Progesterone Receptor and PTEN Expression Predict Survival in Patients with Low and Intermediate Grade Pancreatic Neuroendocrine Tumors. *Arch Pathol Lab Med.* Accepted Sept. 2013, *In press.*
42. Deng L, Feng J, Broaddus RR. The novel estrogen-induced gene EIG121 regulates autophagy and promotes cell survival under stress. *Cell Death Dis.* 2010; 1:e32. [PubMed: 21072319]
43. Kapp AV, Jeffrey SS, Langerod A, et al. Discovery and validation of breast cancer subtypes. *BMC Genomics.* 2006; 7:231. [PubMed: 16965636]
44. Kelloff GJ, Lippman SM, Dannenberg AJ, et al. Progress in chemoprevention drug development: the promise of molecular biomarkers for prevention of intraepithelial neoplasia and cancer--a plan to move forward. *Clin Cancer Res.* 2006; 12:3661–3697. [PubMed: 16778094]

Figure 1.

Differential expression of estrogen receptor (ER) β in pancreatic neuroendocrine tumors (PanNET). A-B PanNET exhibiting strong, diffuse staining for ER β (A: H&E $\times 100$; B: ER β $\times 100$). ER β staining was also seen in acinar cells, duct epithelium and islets of Langerhans (A-B, top left). C. PanNET exhibiting no nuclear staining for ER β ($\times 400$). There is staining of stromal cells, which served as internal positive control.

Table 1

Sequences for real-time quantitative PCR assays

Transcripts	Sequences	Accession no.
18S rRNA	(535+) GAGGGAGCCTGAGAAACGG (602-) GTCGGGAGTGGGTAATTGC (555+) FAM-TACCACATCCAAGGAAGGCAGCAGG	M10098
PR	2689(+) GAGCACTGGATGCTGTTGCT 2754(-) GGCTTAGGGCTTGGCTTTC 2710(+) FAM-TCCCACAGCCATTGGGCGTTC	NM_000926
EIG121	3074(+) CTTGCATAGCACCTTTGCAAG 3135(-) CAGTGGGTGTTGCAGGATG 3096(+) FAM-CTGCGGCGATT TGGGTGCC	NM_020775
IGF-1	146(+) GCAATGGGAAAAATCAGCAG 237(-) GAGGAGGACATGGTGTGCA 217(-) FAM-CTTCACCTTCAAGAAATCACAAAAGCAGCA-BHQ1	M26544
IGF-1R	163(+) CGCAACGACTATCAGCAGCT 238(-) AGATGAGCAGGATGTGGAGGT 189(+) FAM-CCTGGAGAACTGCACGGTGATCGA-BHQ1	NM_000875
sFRP1	720(+) GAGCCGGTCATGCAGTTCT 786() CCTCCGGGAACCTTGTCACA 740(+) FAM-CGGCTTCTACTGGCCGAGATCG-BHQ1	NM_003012
sFRP4	1175(+) GCGCACCACTCGTAGTAATCC 1246() TTCTTGGGACTGGCTGGTT 1202(+) FAM-ACCAAAGGAAAGCCTCCTGCTCC-BHQ1	AF026692

Table 2

Clinicopathologic characteristics of 131 patients with pancreatic neuroendocrine tumors

Characteristics	N (%)
Age	
Mean=56 years (range 18–79 years)	
Gender	
Female	50 (38)
<50 years old	20 (15)
50 years old	30 (23)
Male	81 (62)
Associated syndrome	
MEN1	11 (8)
Other ^a	1 (1)
Functional tumors	22 (17)
Tumor size	
Mean=4.5 cm (range 0.7–20 cm)	
WHO grade	
Low (G1)	72 (55)
Intermediate (G2)	59 (45)
AJCC stage	
IA	22 (17)
IB	13 (10)
IIA	26 (20)
IIB	33 (25)
III	0 (0)
IV	37 (28)

Abbreviation: MEN1, Multiple endocrine neoplasia Type 1.

^aOther, tuberous sclerosis.

Table 3

Correlation between estrogen receptor (ER) β and clinicopathologic parameters in patients with pancreatic neuroendocrine tumors

Characteristics (%)	ER β Category			p value
	Negative (n=5)	Low (n=87)	High (n=39)	
Median age (range)	56 y (49–63)	54 y (22–79)	60 y (18–76)	0.41
Median size (range)	5.0 cm (2.0 – 8.0)	4.0 cm (1.1 – 15.0)	2.6 (0.7 – 20.0)	0.02
Functionality				0.23
Non-functional	4 (4)	76 (70)	29 (26)	
Insulinoma	0 (0)	7 (50)	7 (50)	
Non-insulinoma	1 (13)	4 (50)	3 (37)	
MEN1	1 (9)	5 (45)	5 (45)	0.26
WHO grade				0.02
Low (G1)	5 (7)	41 (57)	26 (36)	
Intermediate (G2)	0 (0)	46 (78)	13 (22)	
AJCC Stage				0.006
I-IIA	1 (2)	34 (55)	26 (43)	
IIB	3 (9)	21 (64)	9 (27)	
IV	1 (3)	32 (86)	4 (11)	

Abbreviations: WHO, World Health Organization; AJCC, American Joint Committee on Cancer

Correlation between estrogen-induced genes and ER β immunohistochemistry in pancreatic neuroendocrine tumors

Table 4

Characteristics	N	Median Estrogen-Induced Genes (mRNA/18S rRNA) $\times 10^4$ (range)					
		PR	EIG121	IGF-I	IGF-1R	sFRP1	sFRP4
ER β Allred score (ρ)	131	0.39	0.30	-0.14	0.17	-0.14	-0.10
<i>P</i> value		<0.001	<0.001	0.10	0.05	0.11	0.28
ER β Category*							
Negative	5	17 (4.9 – 38)	230 (71 – 640)	1.9 (1.0 – 23)	16 (9.6 – 74)	7.9 (1.0 – 16)	34 (2.0 – 320)
Low	87	23 (0 – 450)	350 (9.4 – 1800)	1.6 (0 – 14)	17 (0 – 130)	6.6 (0.2 – 210)	22 (0.1 – 540)
High	39	57 (12 – 540)	490 (79 – 1700)	1.2 (0.1 – 11)	32 (2.6 – 230)	4.0 (0.3 – 106)	17 (0.2 – 280)
<i>P</i> value		<0.001	0.02	0.27	0.06	0.30	0.39

* ER β category: negative = Allred score 0, low = Allred score 3–6, high = Allred score 7–9.

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; EIG121, estrogen-induced gene – 121; IGF, insulin-like growth factor; IGF-1R, insulin-like growth factor-1 receptor; sFRP, secreted frizzled-related protein; ρ , Spearman correlation coefficient.

Table 5

Correlation between estrogen-induced genes and clinicopathologic parameters in normal pancreas and pancreatic neuroendocrine tumors

Characteristics	N	Median Estrogen-Induced Genes (mRNA/18S rRNA) ×10 ⁴ (range)					
		PR	EIG121	IGF-1	IGF-1R	sFRP1	sFRP4
Normal	41	2.2 (0.6 – 14)	110 (41 – 230)	0.8 (0.2 – 18)	22 (8 – 45)	1.3 (0.3 – 9)	1.2 (0.1 – 595)
Tumor	131	32 (0 – 540)	380 (9.4 – 1800)	1.5 (0 – 23)	20 (0 – 230)	6.2 (0.2 – 210)	20 (0.1 – 540)
P value		<0.001	<0.001	0.002	0.84	<0.001	<0.001
Age (p)	131	.05	.05	.02	.02	.06	.03
P value		0.56	0.57	0.85	0.79	0.52	0.71
Gender							
Female <50 years	20	28 (3.4 – 140)	420 (82 – 1800)	1.7 (0.3 – 10)	18 (3.2 – 73)	6.8 (0.4 – 110)	13 (0.2 – 360)
Female 50 years	30	34 (1.5 – 450)	460 (40 – 1400)	1.1 (0.1 – 11)	21 (2.5 – 200)	7.2 (0.2 – 39)	17 (0.2 – 240)
Male	81	31 (0 – 540)	310 (9.4 – 1700)	1.5 (0 – 23)	20 (0 – 230)	5.4 (0.2 – 210)	20 (0.1 – 540)
P value		0.81	0.11	0.39	0.96	0.72	0.97
Tumor size (p)	131	–0.17	0.17	–0.21	–0.18	0.23	–0.26
P value		0.05	0.05	0.01	0.04	0.01	0.002
WHO grade							
Low (G1)	72	43 (1.6 – 350)	360 (9.3 – 1800)	1.2 (0.1 – 23)	25 (3.5 – 230)	4.3 (0.2 – 110)	14 (0.2 – 340)
Intermediate (G2)	59	26 (0 – 540)	400 (40 – 1300)	1.7 (0 – 14)	15 (0 – 130)	8.6 (0.2 – 210)	30 (0.1 – 540)
P value		0.03	0.85	0.05	0.001	0.001	0.24
AJCC Stage							
IA – IIA	61	44 (1.6 – 350)	390 (9.4 – 1700)	1.5 (0.1 – 11)	24 (2.6 – 230)	3.9 (0.3 – 210)	20 (0.2 – 360)
IIB	33	38 (0 – 540)	490 (40 – 1800)	1.6 (0.1 – 23)	20 (2.4 – 200)	6.4 (0.2 – 65)	17 (0.1 – 540)
IV	37	18 (0 – 310)	270 (54 – 1100)	1.6 (0 – 14)	15 (0 – 91)	9.1 (0.2 – 190)	15 (0.2 – 340)
P value		0.003	0.02	0.43	0.005	0.02	0.98

Abbreviations: PR, progesterone receptor; EIG121, estrogen-induced gene – 121; IGF, insulin-like growth factor; IGF-1R, insulin-like growth factor-1 receptor; sFRP, secreted frizzled-related protein; ρ, Spearman correlation coefficient; WHO, World Health Organization; AJCC, American Joint Commission on Cancer.