

# Molecular Markers for Novel Therapeutic Strategies in Pancreatic Endocrine Tumors

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**Objectives:** Pancreatic endocrine tumors (PETs) share numerous features with gastrointestinal neuroendocrine (carcinoid) tumors. Targets of novel therapeutic strategies previously assessed in carcinoid tumors were analyzed in PETs (44 cases).

**Methods:** Activating mutations in *EGFR*, *KIT*, and *PDGFRA* and non-response mutations in *KRAS* were evaluated. Copy number of *EGFR* and *HER-2/neu* was quantified by fluorescence in situ hybridization. Expression of *EGFR*, *PDGFRA*, *VEGFR1*, *TGFBR1*, *Hsp90*, *SSTR2A*, *SSTR5*, *IGF1R*, *mTOR*, and *MGMT* was measured immunohistochemically.

**Results:** Elevated *EGFR* copy number was found in 38% of cases but no *KRAS* non-response mutations. *VEGFR1*, *TGFBR1*, *PDGFRA*, *SSTR5*, *SSTR2A*, and *IGF1R* exhibited the highest levels of expression in the largest percentages of PETs.

Anticancer drugs BMS-754807 (selective for *IGF1R/IR*), 17-(allylamino)-17-demethoxygeldanamycin (17-AAG, targeting *Hsp90*), and axitinib (directed toward *VEGFR1-3/PDGFRB-KIT*) induced growth inhibition of human QGP-1 PET cells with  $IC_{50}$  values (nM) of 273, 723, and 743, respectively. At growth-inhibiting concentrations, BMS-754807 inhibited *IGF1R* phosphorylation; 17-AAG induced loss of *EGFR*, *IGF1R*, and *VEGFR2*; and axitinib increased p21<sup>Waf1/Cip1</sup> (*CDKN1A*) expression without inhibiting *VEGFR2* phosphorylation.

**Conclusions:** Results encourage further research into multidrug strategies incorporating inhibitors targeting *IGF1R* or *Hsp90* and into studies of axitinib combined with conventional chemotherapeutics toxic to tumor cells in persistent growth arrest.

**Key Words:** pancreatic endocrine tumors, molecular analysis

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Pancreatic endocrine tumors (PETs), including islet cell carcinomas, account for 1% to 3% of all pancreatic cancers.<sup>1</sup> An identifying characteristic of some PETs is their overproduction of polypeptide hormones (eg, gastrin, glucagon, insulin, somatostatin, or vasoactive intestinal peptide); these tumors are classified as functional or nonfunctional based on whether their excessive

polypeptide synthesis induces clinical symptoms of hormonal syndromes. An analysis of 1483 cases of PETs in the Surveillance, Epidemiology, and End Results (SEER) database (1973–2000) indicates that, despite the categorization of this neoplasm as an indolent tumor, the median overall survival of patients with metastatic disease is only 17 months.<sup>2</sup> Patients seen at centers specializing in the treatment of neuroendocrine tumors seem to have superior survival when compared with patients in population-based databases like the SEER registry.<sup>3</sup> Complete surgical resection is the most successful treatment for patients with PETs; however, most patients present with advanced stages of disease,<sup>2</sup> for which few current treatment options yield high tumor regression rates. Somatostatin therapy can ameliorate clinical symptoms and perhaps induce tumor growth stabilization (review Modlin et al<sup>4</sup>), and in recent phase III trials, *mTOR* inhibitor everolimus and tyrosine kinase inhibitor (TKI) sunitinib improved progression-free survival<sup>5,6</sup>; however, novel therapeutic strategies exhibiting increased antitumor activity would be beneficial.

Gastrointestinal neuroendocrine (carcinoid) tumors and pancreatic endocrine tumors share a number of characteristics, including neuroendocrine properties (originating from cells of the diffuse neuroendocrine system), certain ultrastructural features (such as cytoplasmic dense-core secretory granules), and similar biomarker expression (eg, chromogranin, synaptophysin, and keratin). Both malignancies can be associated with clinical syndromes induced by tumor overproduction of bioactive peptides or amines which serve as a diagnostic marker for the functioning gastroenteropancreatic endocrine tumor, for example, elevated levels in urine of serotonin catabolite 5-HIAA for carcinoid tumors, serum insulin for insulinoma PETs, serum gastrin for gastrinoma PETs, and serum glucagon for glucagonoma PETs. Patients with carcinoid tumors commonly present at advanced stages for which few treatment options are available; therefore, we recently evaluated a heterogeneous collection of 104 carcinoid tumors for growth factor receptors and downstream effectors and regulators targeted by protein kinase inhibitors and/or antibodies under development as anticancer therapeutics in other forms of cancer.<sup>7</sup> The results were supportive of further research into targeting *Hsp90*, *IGF1R*, and *EGFR* for development of new therapeutic strategies for some carcinoid tumors.

Owing to the similarities shared by gastroenteropancreatic endocrine tumors, the present study investigated in 67 PETs the genetic abnormalities and protein expression of growth factor receptors and downstream regulators examined earlier in carcinoid tumors.<sup>7</sup> The genetic abnormalities analyzed were biomarkers of response to targeted therapeutics developed for other cancers, including activating mutations in *EGFR* exons 18, 19, and 21 and high *EGFR* copy number by fluorescence in situ hybridization (FISH), the former a marker for response to anti-*EGFR* TKI gefitinib in non-small-cell lung cancer,<sup>8,9</sup> and the latter a biomarker predictive of sensitivity to gefitinib in non-small-cell lung cancer<sup>10,11</sup> and to anti-*EGFR* monoclonal antibodies cetuximab and panitumumab in colorectal cancer.<sup>12–14</sup>

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Mutations in *KRAS* codons 12 and 13 were assessed as markers for nonresponse to anti-EGFR therapy, correlating with lack of sensitivity to cetuximab<sup>15–17</sup> and panitumumab<sup>18</sup> in colorectal cancer and to TKIs gefitinib and erlotinib in lung cancer.<sup>19–21</sup> High *HER-2/neu* copy number was measured by FISH as a biomarker predicting response to anti-HER2 monoclonal antibody trastuzumab in breast cancer.<sup>22</sup> Finally, mutations in *KIT* exons 9, 11, 13, and 17 and in *PDGFRA* exons 12, 14, and 18 were analyzed owing to association with sensitivity to TKI imatinib in gastrointestinal stromal tumors.<sup>23,24</sup>

The protein expression analyzed was that of growth factor receptors and downstream effectors and regulators, as measured by immunohistochemistry (IHC). Immunohistochemical expression in PETs of the common therapeutic targets somatostatin receptors SSTR2A and SSTR5 was compared with that of EGFR, PDGFRA, VEGFR1, TGFBR1, Hsp90, IGF1R, and mTOR. Furthermore, the immunohistochemical absence of the demethylating enzyme MGMT was measured as a marker for response of PETs to the DNA-methylating chemotherapeutic temozolomide.<sup>25</sup>

Finally, follow-up in vitro studies were performed in QGP-1 cells, the sole well-established PET cell line, to measure the effect of therapeutics targeting 4 molecular markers that were strongly or moderately strongly expressed immunohistochemically in PETs (VEGFR1, PDGFRA, IGF1R, and Hsp90) and a biomarker with elevated gene copy number by FISH (*EGFR*). The effects on QGP-1 cells of the following anticancer drugs were compared: TKIs axitinib (selective for VEGFR1-3/PDGFR1-3/*KIT*), sunitinib (targeting  $\geq 9$  receptor tyrosine kinases including VEGFR1-3/PDGFR1-3), BMS-754807 (specific for IGF1R/IR), and gefitinib (targeting EGFR), as well as Hsp90 inhibitor 17-(allylamino)-17-demethoxygeldanamycin (17-AAG).

We report here a multifaceted study based on mutational, gene copy number, immunohistochemical, and in vitro analyses, which assessed biomarkers for novel therapeutic strategies in a collection of 41 primary and 26 metastatic PETs, and compared the results with data obtained from neuroendocrine (carcinoid) tumors. This work was presented in preliminary form at the 100th Annual Meeting of the United States and Canadian Academy of Pathology in February 2011.<sup>26</sup>

## MATERIALS AND METHODS

### Patient Samples

Forty-four patients were identified undergoing surgery at Mayo Clinic between 2001 and 2005 for PETs. All cases had accessible pathology slides as well as formalin-fixed, paraffin-embedded tumor blocks, and most had flash-frozen surgical specimens available for analysis. Before inclusion of a case in this study, an hematoxylin and eosin–stained slide from each tumor block associated with the case was reviewed (M.H.M. and R.V.L.) to confirm the PET diagnosis. Written research authorization was obtained from all patients for this study, as well as Mayo Clinic Institutional Review Board approval.

### Tissue Microarray Construction

A tissue microarray (TMA) was constructed by the Tissue and Cell Molecular Analysis Shared Resource, Mayo Clinic, with a Beecher ATA-27 automated arrayer (Sun Prairie, WI). From 44 cases, 67 primary and metastatic PETs were selected. The most characteristic area from each tumor was circled on an hematoxylin and eosin slide, and triplicate 0.6-mm cores were removed from the corresponding area in the associated formalin-fixed, paraffin-embedded tissue block and placed into a single recipient paraffin block. All of the tumor samples selected for

**TABLE 1.** Characteristics of Cases of Pancreatic Endocrine Tumors (44)

Sex	
Male	23
Female	21
Age, median (range) at surgery, y	52 (19–84)
Subtype	
Nonfunctional	38
Functional	
Insulinoma	4
Gastrinoma	1
Glucagonoma	1
Previous therapy	5
History of other cancer	9
Tumor sites in TMA*	
Pancreas	41
Liver	16
Lymph node	7
Duodenum	2
Diaphragm	1

\*TMA constructed with 41 PET primaries and 26 metastatic PETs.

constructing the TMA are listed, by case, in Supplementary Table 1 (Supplemental Digital Content 1, <http://links.lww.com/MPA/A198>).

### Immunohistochemical Analysis

Sections (5  $\mu$ m) of the PET TMA were analyzed by IHC for EGFR, PDGFRA, VEGFR1, mTOR, IGF1R, Hsp90, TGFBR1, MGMT, SSTR2A, and SSTR5. Immunohistochemical staining was performed by the Tissue and Cell Molecular Analysis Shared Resource, Mayo Clinic. Positive controls for IHC stains were normal colon (for TGFBR1), normal pancreas (SSTR2A and SSTR5), breast cancer (EGFR, IGF1R, and PDGFRA), normal skin (VEGFR1), prostate cancer (mTOR and Hsp90), and colon cancer (MGMT). Negative controls for all stains were prepared by substituting diluent for primary antibodies. IHC of all biomarkers was scored based on intensity by 2 pathologists (R.V.L. and L.J.A.), with a score of 0 indicating absence of staining and 1, 2, and 3 representing weak, moderate, and strong staining intensity, respectively. The immunohistochemical intensity score reported for the staining of tumor cells within each assessable PET was the average from the replicate TMA cores for that sample. All immunohistochemical antibodies and epitope retrieval methods are listed in Supplementary Table 2 (Supplemental Digital Content 2, <http://links.lww.com/MPA/A199>).

### Fluorescence In Situ Hybridization Analysis of Gene Copy Number

PET TMA sections (5  $\mu$ m) were analyzed by FISH for *HER-2/neu* and *EGFR* copy number by the Cytogenetics Shared Resource Laboratory, Mayo Clinic, by the methodology previously reported.<sup>27</sup> Briefly, 30 nuclei were scored per sample with quantitation of red signals (*HER-2/neu* or *EGFR*) and green signals (chromosome 17 centromere [CEP17] or chromosome 7 centromere [CEP7], respectively). A *HER-2/neu*–to–CEP17 ratio or *EGFR*–to–CEP7 ratio of 0.8 to 1.30 was interpreted as normal, less than 0.8 as gene deletion, 1.30 to 2.0 as gene duplication, and 2.0 or greater as gene amplification. Aneusomy was defined to be a normal *HER-2/neu*–to–CEP17 or *EGFR*–to–CEP7 ratio with greater than 30% of cells having 3 or more

TABLE 2. Pancreatic Endocrine Tumors Exhibiting Strong Biomarker Expression

Case	WHO 2004 classification	Tumor site	VEGFRI	TGFBRI	PDGFRA	SSTR5	SSTR2A	IGFIR	Hsp90	EGFR	mTOR	MGMT	Mean EGFR ≥2.47/cell
1	2*	Pancreas	+	-	-	-	-	-	-	-	-	-	-
2†	2	Liver	+	+	+	+	+	+	-	-	-	-	+
3	1.2	Lymph node	+	+	+	+	+	+	-	-	-	-	+
4	2	Liver	+	+	+	+	+	+	+	+	-	-	+
5§	2	Pancreas	+	+	+	+	+	+	+	+	-	-	+
6	1.2	Pancreas	-	-	-	-	-	-	-	-	-	-	-
7	2	Diaphragm	+	+	+	+	+	+	-	-	-	-	-
8	2	Pancreas	+	+	+	+	+	+	-	-	+	-	-
9	2	Liver	+	+	+	+	+	+	-	-	-	-	-
10	2	Pancreas	+	+	+	+	+	+	-	-	-	-	-
11	2	Lymph node	+	+	+	+	+	+	-	-	-	-	-
12	2	Pancreas	+	+	+	+	+	+	-	-	-	-	-
13#	2	Pancreas	+	+	+	+	+	+	-	-	-	-	-
14	1.2	Liver	+	+	+	+	+	+	+	+	-	-	+
15	2	Pancreas	+	+	+	+	+	+	-	-	-	-	+
16	1.2	Liver	+	+	+	+	+	+	-	-	-	-	+
17	1.2	Pancreas	+	+	+	+	+	+	-	-	-	-	+
18#	1.2	Pancreas	+	+	+	+	+	+	+	+	-	-	+
19	1.2	Lymph node	+	+	+	+	+	+	+	+	-	-	+
20*	2	Pancreas	+	+	+	+	+	+	-	-	-	-	+
21	1.2	Pancreas	+	+	+	+	+	+	-	-	-	-	+
22#**	1.1	Pancreas	+	+	+	+	+	+	-	-	-	-	+
23	1.2	Pancreas	+	+	+	+	+	+	-	-	-	-	+
24#	1.2	Pancreas	+	+	+	+	+	+	-	-	-	-	+

(Continued on next page)

TABLE 2. (Continued)

Case	WHO 2004 classification	Tumor site	VEGFR1	TGFBRI	PDGFRA	SSTR5	SSTR2A	IGF1R	Hsp90	EGFR	mTOR	MGMT	Mean EGFR $\geq 2.47$ /cell
25	1.2	Pancreas	+	-	+	+	+	+	-	-	-	-	+
26	2	Pancreas	-	-	-	+	-	+	-	-	-	-	-
		Liver	+	+	-	+	+	-	-	-	-	-	-
27	2	Pancreas	+	-	+	-	+	+	-	-	-	-	-
		Duodenum	+	+	+	+	+	+	-	-	-	-	+
28*	2	Liver	+	+	-	+	-	-	+	-	-	-	+
29	2	Pancreas	-	+	+	-	-	-	+	-	-	-	-
		Liver	-	+	-	-	-	+	-	-	-	-	-
30	1.2	Pancreas	-	-	-	+	-	-	-	-	-	-	-
31	2	Pancreas	+	+	+	+	+	-	+	-	-	-	-
		Liver	+	+	+	+	+	-	-	-	-	-	+
32	2	Pancreas	+	+	-	-	-	-	-	-	-	-	-
		Liver	-	-	-	-	-	+	-	-	-	-	-
33**††	2	Pancreas	+	+	+	+	+	+	-	-	-	-	-
		Duodenum	+	+	+	+	-	-	-	-	-	-	-
34	2	Pancreas	+	+	+	+	-	+	+	-	-	-	-
35	2	Pancreas	+	-	+	-	-	+	-	-	-	-	-
36†††	2	Pancreas	+	+	+	-	-	-	-	-	-	-	-
		Lymph node	+	+	+	+	+	-	-	-	-	-	-
		Liver	+	+	-	+	-	-	-	+	-	-	-
37	1.2	Pancreas	+	+	-	+	+	-	-	-	-	-	-
38	1.2	Pancreas	+	+	+	-	-	-	+	-	-	-	-
39	1.1	Pancreas	+	+	+	+	+	+	-	-	-	-	-
40	1.2	Pancreas	+	-	+	-	-	+	-	-	-	-	-
41	1.2	Pancreas	+	+	+	-	+	+	-	-	-	-	+
42	1.2	Pancreas	+	+	+	-	+	+	-	-	-	-	+
43	2	Pancreas	+	-	-	-	-	-	-	-	-	-	+
44	2	Liver	+	-	+	+	+	-	-	-	-	-	+

\*1.1, well-differentiated endocrine tumor with "benign behavior"; 1.2, well-differentiated endocrine tumor with uncertain behavior; 2, well-differentiated endocrine carcinoma.  
 †For VEGFR1, TGFBRI, PDGFRA, SSTR5, SSTR2A, IGF1R, Hsp90, EGFR, mTOR, and MGMT, plus (+) indicates an IHC intensity score of 3, and minus (-) indicates an IHC score less than 3; for MGMT, em dash (-) indicates a score of 0.  
 ‡Tumor(s) FISH-positive for EGFR and HER-2/neu.  
 §PET therapy before this instance of surgery was radiation therapy.  
 ¶PET therapy before this instance of surgery was somatostatin analog therapy.  
 ¶PET therapy before this instance of surgery was chemotherapy.  
 #Functioning PET is insulinoma.  
 \*\*PET therapy before this instance of surgery was surgery.  
 ††Functioning PET is gastrinoma.  
 †††Functioning PET is glucagonoma.

CEP17 or CEP7 signals, respectively (ie, a balanced gain in the number of gene copies and the number of chromosome copies). Aneuploid tumors were analyzed for mean *EGFR* copy number/cell to determine whether *EGFR* copy number was elevated ( $\geq 2.47$  per nucleus<sup>13</sup> or  $\geq 2.92$  per cell<sup>14</sup>). *HER-2/neu* and *EGFR* FISH-positive samples were those demonstrating amplification or high aneusomy with 40% or greater of cells having 4 or more copies of *HER-2/neu* or *EGFR*, respectively. Owing to the inherent admixture of tumor and nontumor cells within PETs, FISH-positivity for *HER-2/neu* and *EGFR* as well as FISH-determined elevation of *EGFR* copy number/cell was assigned the highest value score from the replicate TMA cores for each assessable neoplasm.

### Mutational Analysis of Selected *EGFR*, *KIT*, *PDGFRA*, and *KRAS* Exons

Thirty-six surgical specimens from the 44 cases in this study were flash-frozen after excision and stored at  $-70^{\circ}\text{C}$  by the Tissue Core Resource of the Mayo Clinic Cancer Center SPORE in Pancreatic Cancer. DNA for mutational analyses was isolated from frozen tissue samples with the QIAamp DNA Mini Kit (Qiagen, Valencia, CA). Mutational analyses of *EGFR* (exons 18, 19, and 21) and *KIT* (exons 9, 11, 13, and 17) were performed as reported (Gilbert et al<sup>27,28</sup>); mutational analyses of *KRAS* (exon 2) and *PDGFRA* (exons 12, 14, and 18) were conducted as described.<sup>7</sup> Amplification and sequencing were repeated for all observed polymorphisms and mutations. All tissues assessed for *EGFR*, *KIT*, *PDGFRA*, and *KRAS* mutations are listed by case in Supplementary Table 1 (Supplemental Digital Content 1, <http://links.lww.com/MPA/A198>).

### Cell Culture

The QGP-1 (monolayer) PET cell line was purchased from the Japan Health Sciences Foundation's Health Science Research Resources Bank (Osaka, Japan). The QGP-1 line was established from a human pancreatic islet cell carcinoma lesion in the late 1970s, exhibiting morphological and histological similarities to the original tumor.<sup>29</sup> The NCI-H727 (monolayer) human bronchopulmonary neuroendocrine tumor (carcinoid) cell line was purchased from American Type Culture Collection (Manassas, VA). Cell lines were cultured at  $37^{\circ}\text{C}$  in a humidified environment of 95%:5% air-to- $\text{CO}_2$  in RPMI 1640 media (Invitrogen; Carlsbad, CA) supplemented with 10% vol/vol heat-inactivated fetal bovine serum (PAA Labs; New Bedford, MA).

### Cell Proliferation Assay

QGP-1 (4000 per well) or NCI-H727 cells (4000 per well) were seeded into 96-well plates (Corning, Corning, NY) in 100  $\mu\text{L}$  aliquots of growth medium and incubated ( $37^{\circ}\text{C}$ ) for 48 hours. Each drug concentration was added in 100  $\mu\text{L}$  of growth medium containing 0.125% vol/vol DMSO to 6 replicate wells; 100  $\mu\text{L}$  of growth medium containing 0.125% vol/vol DMSO without drug was added to 6 replicate wells of control cells. After incubation for 5 days (continuous drug exposure), cell growth was determined with the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay<sup>30</sup> upon addition to each well of 40  $\mu\text{L}$  containing a 20:1 ratio of 2-mg/mL MTS (Promega, Madison, WI) and 0.92 mg/mL phenazine methosulfate (Sigma, St. Louis, MO). After incubation for 2 hours, absorbance at 490 nm was measured on a SpectraMax Plus<sup>384</sup> (Molecular Devices, Sunnyvale, CA) microplate reader. Dose response experiments for each drug were performed in triplicate, with growth of treated cells at each dosage compared with proliferation of control cells.  $\text{IC}_{50}$  values were estimated with Prism software (GraphPad, San Diego,

CA) using nonlinear least squares regression analysis to fit the mean percent control value for each dosage to the best sigmoidal dose-response curve. Anticancer drugs axitinib, sunitinib, gefitinib, and BMS-754807 were purchased from ChemiTek (Indianapolis, IN); 17-AAG was provided by the National Cancer Institute (Bethesda, MD).

### Western Immunoblot Analysis

Whole-cell lysates were prepared from cultured cells with RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA) according to manufacturer's instructions; protein was quantitated using the DC Protein Assay (Bio-Rad, Hercules, CA). Western blot analysis was performed after electrophoresis of samples that were loaded onto 10% or 12% wt/vol separating gels (Criterion XT; Bio-Rad) on the basis of protein concentration; after transfer to PVDF membranes (Bio-Rad), immunoreactive proteins were detected using SuperSignal enhanced chemiluminescent substrates (Pierce, Rockford, IL) and Hyblot CL film (Denville Scientific, Metuchen, NJ). Primary antibodies used were anti-EGFR (Santa Cruz sc-03), anti-IGF1R beta (Cell Signaling 3027; Danvers, MA), anti-phospho-IGF1R beta (Tyr1131)/Insulin Receptor beta (Tyr1146) (Cell Signaling 3021), anti-VEGFR2 (Cell Signaling 2479), anti-phospho-VEGFR2 (Tyr1059) (Cell Signaling 3817), anti-p21/Waf1/Cip1 (CDKN1A) (Cell Signaling 2947), anti-Hsp90 (Cell Signaling 4874), and anti- $\alpha$ / $\beta$ -tubulin (Cell Signaling 2148); the secondary antibody was goat anti-rabbit IgG-HRP (Santa Cruz sc-2004).

## RESULTS

### Patient Cases

Of 44 patients in this study, 21 were female and 23 were male (Table 1). The median age at the time of surgery was 52 years (range, 19–84).

Most of the patients (38) had nonfunctional PETs, and 5 patients had received treatment of pancreatic endocrine tumors before surgery (cases identified in Table 2). The classification of all cases according to the World Health Organization 2004 clinicopathological criteria<sup>31</sup> is provided in Table 2.

Sixty-seven PETs (Table 1) from the 44 cases were accessible in formalin-fixed, paraffin-embedded blocks and were included in the TMA construction, with liver the most frequent metastatic site represented. Supplementary Table 1 (Supplemental Digital Content 1, <http://links.lww.com/MPA/A198>) lists all PETs, by case, included in the TMA.

### Mutational Analysis of *EGFR*, *KIT*, *PDGFRA*, and *KRAS*

Thirty-six PETs (from 35 of the 44 cases) were available as frozen specimens, providing DNA for analysis of mutations in selected exons of *EGFR*, *KIT*, *PDGFRA*, and *KRAS*. Supplementary Table 1 (Supplemental Digital Content 1, <http://links.lww.com/MPA/A198>) lists, by case, the tissues (all primary tumors except one) examined by mutational analysis.

No PET analyzed displayed *KRAS* exon 2 mutations that are associated with nonresponse to anti-EGFR monoclonal antibodies<sup>15–18</sup> and TKIs.<sup>19–21</sup> No PET exhibited *EGFR* mutations predictive of gefitinib sensitivity, no *KIT* or *PDGFRA* mutations associated with clinical response to imatinib.

Polymorphisms in *PDGFRA* and *EGFR* were detected in some PETs during mutational analyses. All tumors analyzed were homozygous for the synonymous single nucleotide polymorphism (SNP) dbSNP rs1873778 in *PDGFRA* exon 12 (A1701G, where number "1" is assigned to the "A" in the translation initiation codon of the cDNA; amino acid position 567). Pancreatic



endocrine tumors from 16% of the cases examined were heterozygous for the synonymous SNP dbSNP rs2228230 in *PDGFRA* exon 18 (C2472T; amino acid 824). In addition, tumor in 2 cases exhibited heterozygosity for the synonymous SNP dbSNP rs2229066 in *EGFR* exon 21 (C2508T; amino acid 836).

### Fluorescence In Situ Hybridization Analysis of *EGFR* and *HER-2/neu* Copy Number

#### *EGFR*

Fluorescence in situ hybridization analysis determined that some of the assessable cases had PETs displaying *EGFR* aneusomy with *EGFR* copy number at elevated levels associated with the response of colorectal cancer to panitumumab and cetuximab: 38% exhibited 2.47 or greater per nucleus, 28% exhibited 2.92 or greater per cell. Of the 19 cases in which multiple tumor tissues from the same patient were assessable for elevated *EGFR* copy number, all sites were positive in 4 cases and all negative in 13. Table 2 presents the PETs exhibiting elevated *EGFR* copy number, on a case-to-case basis.

Three cases with PETs displaying elevated *EGFR* copy number were also determined to be “FISH-positive,” with *EGFR* copy number at high levels associated with gefitinib response (aneusomy with  $\geq 40\%$  of cells having  $\geq 4$  copies of *EGFR*<sup>10,11</sup>). *EGFR* FISH-positive tumors, all from cases with nonfunctional PETs and all tumors from each case exhibiting FISH-positivity, are indicated in Table 2.

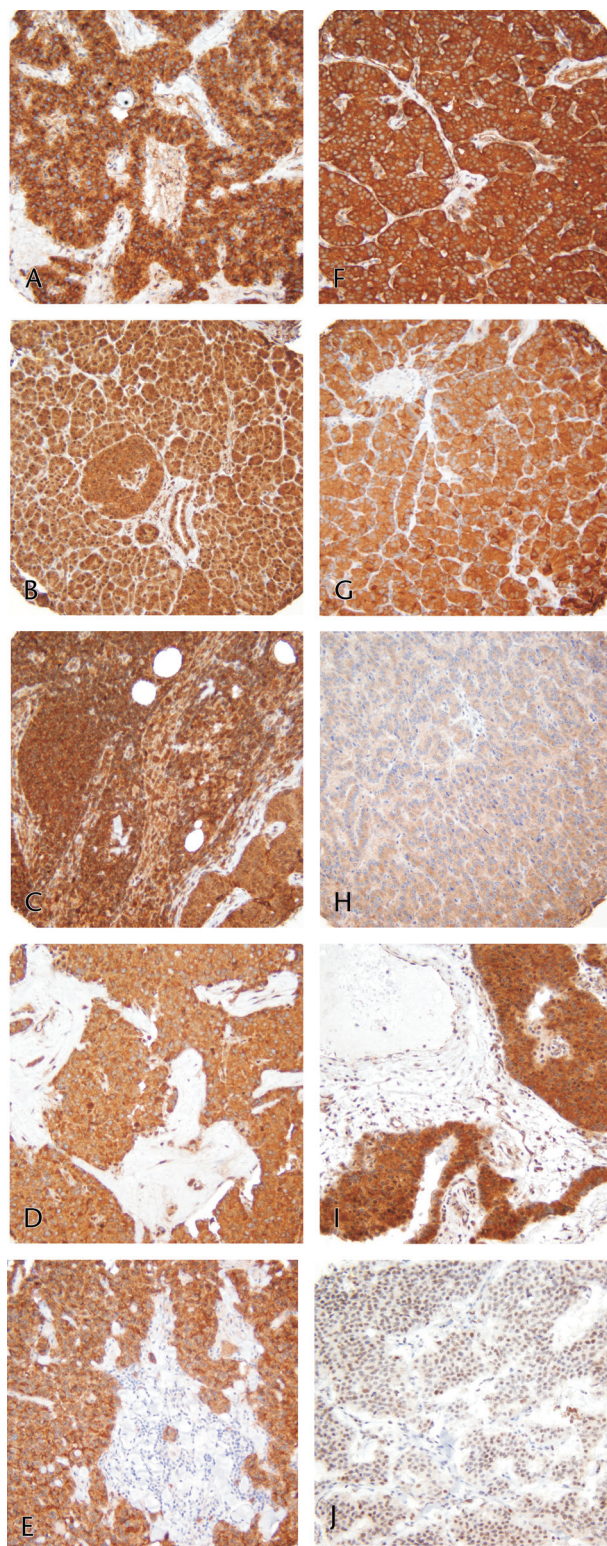
#### *HER-2/neu*

*HER-2/neu* aneusomy was detected in 33% of the assessable cases, although PET *HER-2/neu* copy number was below levels predictive of anti-HER2 response (defined as  $>6$  *HER-2/neu* copies per cell by FISH analysis<sup>32</sup>). However, 3 cases with *HER-2/neu* aneusomy displayed high copy number for both *EGFR* and *HER-2/neu* (aneusomy with  $\geq 40\%$  of cells having  $\geq 4$  gene copies), a combination predictive of gefitinib sensitivity greater than that for patients with high *EGFR* copy number alone.<sup>33</sup> Table 2 provides the cases with tumors that were FISH-positive for both *HER-2/neu* and *EGFR*, all well-differentiated endocrine carcinomas.

### Immunohistochemical Analysis

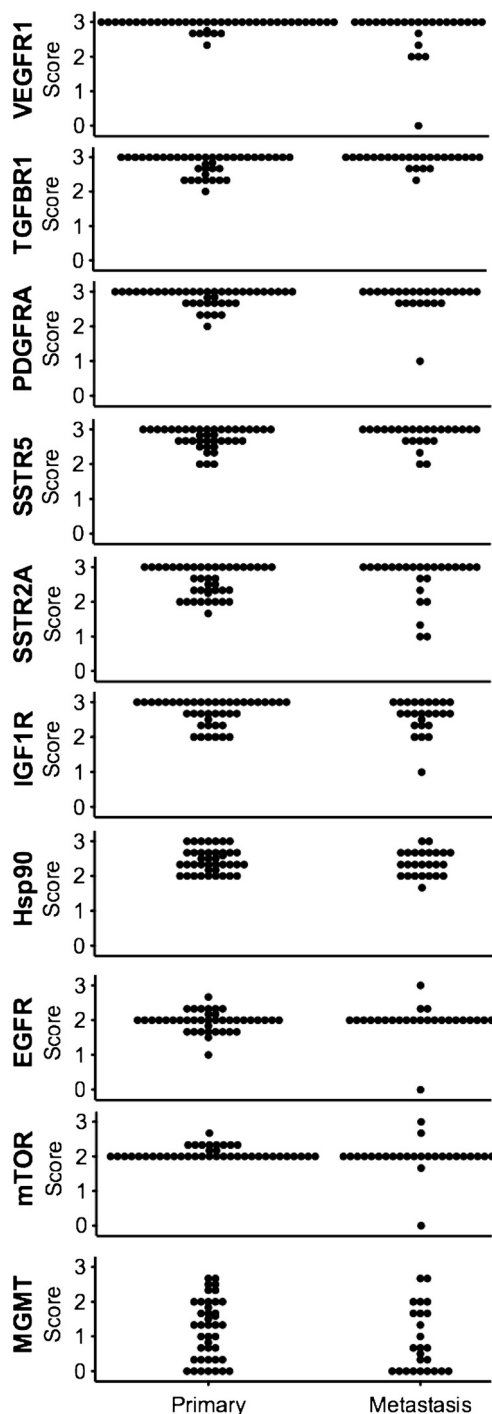
The immunohistochemical staining of pancreatic endocrine tumors for SSTR2A, SSTR5, EGFR, PDGFRA, VEGFR1, TGFBR1, Hsp90, IGF1R, mTOR, and MGMT is illustrated in Figure 1. All of the immunohistochemical stains showed a diffuse cytoplasmic pattern with the exception of MGMT, which was a nuclear stain, and SSTR2A, which was predominantly membranous. IGF1R, mTOR, and SSTR5 in most cases stained in a diffuse pattern; however, there were a few cases that had more of a nuclear staining pattern with weak cytoplasmic staining; the significance of these findings was uncertain. Predominantly focal staining was not noted with any of the antibodies. A PET sample was assigned a score of 1 if the tissue had weak staining diffusely or only a few cells with weak staining.

The immunohistochemical staining intensity score for each assessable PET in the TMA is presented for each biomarker in Figure 2, with scores grouped by location of malignancy (primary or metastasis). With the exception of MGMT, all biomarkers were expressed in all tumors except for 1 liver metastasis with no detectable VEGFR1, EGFR, or mTOR staining. The biomarkers for which the largest number of PETs exhibited the strongest IHC staining were VEGFR1, TGFBR1, PDGFRA, SSTR5, SSTR2A, and IGF1R, with intensity scores of 3 for



**FIGURE 1.** Immunohistochemical analysis of pancreatic endocrine tumors. Individual PET specimens in the TMA (original magnification  $\times 200$ ) exhibiting strong immunostaining for VEGFR1 (A), TGFBR1 (B), PDGFRA (C), SSTR5 (D), SSTR2A (E), IGF1R (F), Hsp90 (G), EGFR (H), mTOR (I), and MGMT (J).

80%, 69%, 65%, 55%, 55%, and 47% of all tumors, respectively. The biomarker with the lowest immunohistochemical expression level was MGMT, with complete absence of staining in 24% of PETs (32% of cases); in 8 of these cases with available primary as well as metastatic tumor tissues, only 1 exhibited



**FIGURE 2.** Immunohistochemical staining intensity of 10 biomarkers in PETs. Individual IHC staining intensity scores for all PETs assessable in the TMA (n = 67) were grouped for comparison by site of malignancy (primary or metastasis).

**TABLE 3.** Anticancer Drugs That Inhibited QGP-1 Human PET Cell Proliferation

Inhibitor	Specificity	IC <sub>50</sub> (nM)*
BMS-754807	IGF1R/IR	273
17-AAG	Hsp90	723
Axitinib	VEGFR1-3, PDGFRA-B, KIT	743
Sunitinib	VEGFR1-3, PDGFRA-B, KIT, FLT3, CSF1R, RET	2707
Gefitinib	EGFR	24,080

\*Cell proliferation was determined by the MTS assay; data were the average of 3 experiments or more.

MGMT deficiency across all sites. For each target protein analyzed immunohistochemically, the staining intensity score averaged over all primaries was not significantly different from the score averaged over all metastases (data not shown) except for VEGFR1, with a mean (SEM) of 2.94 (0.02) for primaries and 2.72 (0.13) for metastases ( $P < 0.05$ ).

Table 2 summarizes, by case, the combination of molecular markers that were strongly expressed (immunohistochemical staining intensity score of 3) in each assessable PET. The percentage of cases with assessable primary as well as metastatic tumors in which all sites exhibited the same biomarker level, that is, an IHC intensity score of either 3 or less than 3 (for MGMT, either <3 or 0), were as follows: For biomarkers EGFR and mTOR, 95%; TGFBR1 and Hsp90, 74%; VEGFR1 and SSTR2A, 68%; MGMT, 63%; SSTR5, 58%; PDGFRA, 47%; and IGF1R, 37%. Of cases in which multiple tumor tissues from the same patient were analyzed, a majority exhibited strong immunohistochemical expression across all sites for biomarkers VEGFR1 and TGFBR1 (58% and 58%, respectively). Most PETs that exhibited strong expression of VEGFR1 also displayed strong staining for PDGFRA (75%), a similar occurrence for SSTR5 and SSTR2A (72%). Of note, strong EGFR immunohistochemical staining was not exhibited by any PET that displayed elevated *EGFR* copy number.

**In Vitro Effects of Anticancer Drugs in PET Cells**

A dearth of well-established human PET cell lines currently exists.<sup>4</sup> Accordingly, our in vitro PET studies were constrained to 1 PET line, QGP-1,<sup>29</sup> for measuring the effects on cellular proliferation and downstream functionality of anticancer drug targeting biomarkers identified in a collection of PETs. The MTS assays performed with QGP-1 cells determined that growth inhibition was produced by increasing concentrations of the following therapeutic agents: axitinib and sunitinib (TKIs targeting VEGFR1-3 and PDGFRA-B, among others), BMS-754807 (a TKI specific for IGF1R and IR), gefitinib (a TKI selective in bronchopulmonary NET cell lines<sup>7</sup>). Table 3 presents IC<sub>50</sub> values for the inhibition of QGP-1 proliferation induced by each of these compounds.

The 3 most potent growth inhibitors, BMS-754807, 17-AAG, and axitinib, were assessed in follow-up experiments with QGP-1 cells to measure functional effects downstream of their targeted proteins, with results from QGP-1 cells compared, in turn, with those obtained in the human NCI-H727 bronchopulmonary neuroendocrine tumor (carcinoid) cell line. Western immunoblot analysis indicated that exposure of QGP-1 cells (24 hours) to BMS-754807 concentrations increasing from 0.1 to 10 μmol/L resulted in

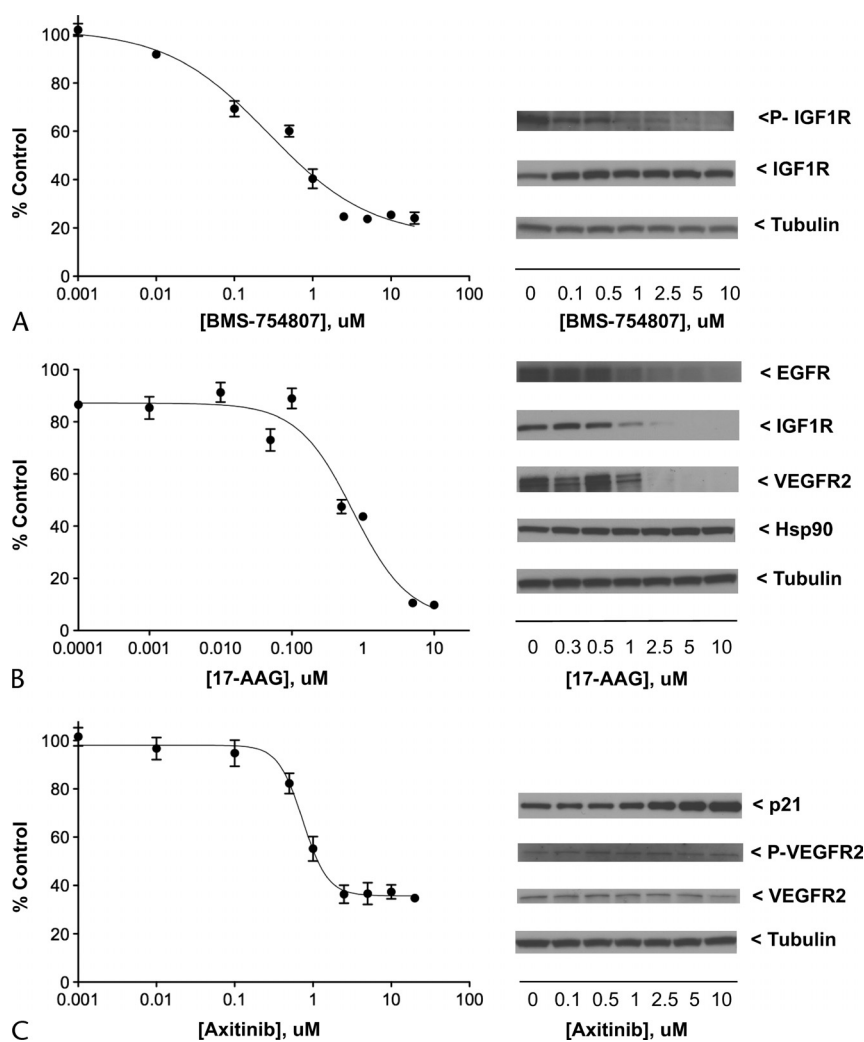


increasing inhibition of the constitutive phosphorylation of IGF1R (n = 3). Figure 3A illustrates the inhibition induced by increasing BMS-754807 concentrations on QGP-1 cell growth as well as on constitutive IGF1R phosphorylation. In comparison, BMS-754807 was less potent at inhibiting NCI-H727 carcinoid cell growth (IC<sub>50</sub> of 428 nmol/L) but reduced constitutive phosphorylation of IGF1R in the NCI-H727 line in a similar fashion (see Gilbert et al<sup>7</sup>).

Incubation of QGP-1 cells (24 hours) with 17-AAG concentrations ranging from 0.3 to 10 μmol/L resulted in dose-dependent decreases in the levels of the client proteins EGFR, IGF1R, and VEGFR2 (the principal mediator of VEGF signaling [review Roskoski et al<sup>34</sup>]) as measured by Western analysis (n = 3). Figure 3B illustrates in QGP-1 cells the 17-AAG-

induced dose-dependent decrease in cell proliferation and in cellular levels of EGFR, IGF1R, and VEGFR2. In comparison, 17-AAG, with 10-fold greater antiproliferative activity in the NCI-H727 carcinoid line (IC<sub>50</sub> of 70.4 nmol/L), decreased in a dose-related fashion the levels in NCI-H727 cells of EGFR and IGF1R [see Gilbert et al<sup>7</sup>] but not VEGFR2 (data not shown).

Western blot analysis determined that QGP-1 cells exposed (24 hours) to axitinib concentrations from 0.1 to 10 μmol/L exhibited increasing levels of p21<sup>Waf1/Cip1</sup> (CDKN1A) (p21) expression with no change in constitutive VEGFR2 phosphorylation (n = 3). Figure 3C presents data on the growth inhibition, increased p21 expression, and constant level of constitutive VEGFR2 phosphorylation seen in QGP-1 cells exposed to axitinib in a dose-related fashion. In contrast, although sunitinib was



**FIGURE 3.** Effects of anticancer drugs in QGP-1 PET cell line. A, left: QGP-1 cells (4000 per well) were incubated (continuous exposure, 5 days) in 96-well plates at 37°C with increasing concentrations of anticancer drug BMS-754807 (targeting IGF1R/IR) in serum-containing medium, with cell proliferation measured by the MTS assay; right: QGP-1 cells (500,000 per well) were incubated (continuous exposure, 24 hours) in 6-well plates at 37°C with increasing concentrations of BMS-754807 in serum-containing medium, with effect on constitutive IGF1R phosphorylation determined by Western immunoblotting of equal quantities of protein from whole cell lysates. B, Effect of increasing concentrations of anticancer drug 17-AAG (targeting Hsp90) on QGP-1 cell growth and levels of indicated biomarkers, with experiments performed as described in A. C, Effect of increasing concentrations of anticancer therapeutic axitinib (targeting VEGFR1-3, PDGFRA-B, and KIT) on QGP-1 cell growth and levels of indicated biomarkers, with experiments performed as described in A. MTS data presented were the mean (SE) of 3 experiments; Western immunoblotting results were representative blots from 1 of 3 experiments.



moderately less potent in NCI-H727 cells ( $IC_{50}$  of 3.24  $\mu\text{mol/L}$ ;  $n = 4$ ), axitinib had little effect on the proliferation of the NCI-H727 carcinoid line. An axitinib concentration of 10  $\mu\text{mol/L}$  inhibited NCI-H727 growth less than 50% ( $n = 3$ ), the solubility limit in RPMI preventing an accurate  $IC_{50}$  determination.

In analyzing the PET cells for genetic abnormalities, *EGFR* aneusomy was detected by FISH in the QGP-1 line, with elevated *EGFR* copy number predictive of sensitivity to panitumumab and cetuximab; these results were found in NCI-H727 carcinoid cells as well (see Gilbert et al<sup>7</sup>). QGP-1 cells were also *EGFR* FISH-positive, with high aneusomy associated with gefitinib response, as were NCI-H727 cells (see Gilbert et al<sup>7</sup>). Mutational analyses detected no *EGFR*, *KIT*, or *PDGFRA* activating mutations in either the QGP-1 or NCI-H727 line. However, QGP-1 (as well as NCI-H727) cells were homozygous for the dbSNP rs1873778 in *PDGFRA* exon 12; the NCI-H727 line was heterozygous for the dbSNP rs2228230 in *PDGFRA* exon 18. Furthermore, in contradistinction to the reported absence in QGP-1 cells of mutations in *KRAS* codons 12 and 13 associated with nonresponse to anti-EGFR therapy,<sup>35</sup> our analysis indicated that QGP-1 cells harbored the *KRAS* nonresponse codon 12 mutation G35T (encoding G12V); NCI-H727 cells also exhibited this mutation, which was heterozygous in both lines. Consistent with the *KRAS* mutational status of QGP-1 cells, gefitinib exhibited the lowest antiproliferative activity of the therapeutic agents analyzed in the QGP-1 line, with a 9- to 90-fold higher  $IC_{50}$  value (Table 3). In NCI-H727 cells, increasing concentrations of gefitinib inhibited proliferation in a dose-related, biphasic-like fashion, with gefitinib concentrations of 5  $\mu\text{mol/L}$  or greater inhibiting growth more than 50% ( $n = 6$ ). Of note, the nonresponse *KRAS* mutation detected in QGP-1 PET cells was absent from all PET tissues analyzed.

Finally, immunohistochemical analysis of QGP-1 cells for the 10 molecular markers evaluated in PET TMA sections determined that strong immunohistochemical staining (IHC staining intensity score of 3) was exhibited by all but 3 biomarkers: MGMT and Hsp90 (intensity score of 2) and SSTR2A (score of 1). In the NCI-H727 line, all molecular markers were strongly expressed except MGMT (score of 2). Interestingly, the biomarkers that exhibited the strongest IHC staining in the largest number of PETs (VEGFR1, TGFBR1, PDGFRA, SSTR5, SSTR2A, and IGF1R) were all strongly expressed in QGP-1 PET cells as well, with the exception of SSTR2A.

## DISCUSSION

Of 1483 cases of PETs diagnosed and recorded in the SEER registry from 1973 to 2000, 80.9% of the patients presented with metastatic or regionally advanced tumors.<sup>2</sup> Although somatostatin therapy is effective for clinical symptoms and may induce tumor growth stabilization (review Modlin et al<sup>4</sup>), PETs generally have a short-lived duration of response to conventional chemotherapeutic agents (review Metz and Jensen<sup>36</sup>); while recent advances have been made in significantly prolonging progression-free survival with everolimus and sunitinib,<sup>5,6</sup> new therapeutic strategies, especially strategies inducing higher radiographic tumor response rates, are needed. We analyzed a collection of PETs for genetic abnormalities and protein expression of a variety of growth factor receptors and downstream effectors and regulators targeted by therapeutics developed for other forms of cancer and assessed previously in a heterogeneous collection of 104 neuroendocrine (carcinoid) tumors.<sup>7</sup>

From analyses for genetic abnormalities in PETs, the *EGFR* FISH assay provided results of note. Elevated *EGFR* copy number was detected in 38% of all assessable cases,

whereas moderate EGFR expression was displayed immunohistochemically in most of the PETs. In comparison, the first immunohistochemical study of both EGFR and activated-EGFR in a heterogeneous collection of metastatic PETs (48 cases) reported EGFR expression in only 25% of PET primaries and 18% of metastatic PETs; these values were 23% and 32%, respectively, for phosphorylated-EGFR expression.<sup>37</sup> Interestingly, 73% of the cases with elevated *EGFR* copy number in this report were well-differentiated endocrine carcinomas; these results, together with the absence of *KRAS* nonresponse mutations in PETs, suggested further research into the role of anti-EGFR monoclonal antibodies for selected PETs.

Protein expression of 10 biomarkers was compared immunohistochemically, including that for SSTR2A and SSTR5. Of the 5 human somatostatin receptor subtypes, SSTR2 and SSTR5 have the highest binding affinity for somatostatin analogs used clinically for gastroenteropancreatic neuroendocrine tumors (primarily octreotide and lanreotide) (review Patel<sup>38</sup>). The high expression levels of SSTR2A (the long form of SSTR2) and SSTR5 in most of the PETs were consistent with the therapeutic importance of somatostatin analogs in PET disease for improving clinical symptoms and potentially stabilizing tumor growth. Although somatostatin analogs have been shown to improve time to progression in carcinoid tumors,<sup>39</sup> no similar studies in patients with PETs have been performed. Despite the lack of clinical data, somatostatin analogs are frequently used in patients with PETs.

In contrast, MGMT displayed the lowest immunohistochemical expression level of 10 biomarkers analyzed in this collection of 67 PETs, with 24% exhibiting no detectable staining. MGMT deficiency, a predictive marker for PET sensitivity to temozolomide,<sup>25</sup> was less common in this study than in an earlier report of 37 archival PETs in which 51% were MGMT deficient.<sup>25</sup>

Our results indicated that, aside from the prevalence of somatostatin receptors in both malignancies, PETs exhibited a different immunohistochemical expression profile for growth factor receptor and downstream regulators than did a heterogeneous collection of neuroendocrine (carcinoid) tumors (104 samples consisting predominantly of bronchopulmonary (21%) and small bowel (17%) primaries as well as liver (29%) and lymph node (10%) metastases<sup>7</sup>). The biomarkers for which the largest number of PETs exhibited the strongest IHC staining were, in decreasing order, VEGFR1, TGFBR1, PDGFRA, and IGF1R as compared with the sequence Hsp90, TGFBR1, and IGF1R in carcinoid tumors (Hsp90 and IGF1R in small bowel primaries alone).<sup>7</sup> The expression of high levels of numerous growth factor receptors and regulators in large percentages of PETs encouraged follow-up in vitro studies of the effects on PET cells of anticancer drugs targeting these biomarkers.

QGP-1 cell growth was inhibited by anticancer drug BMS-754807 (an anti-IGF1R/IR TKI currently in phase I clinical trials for treatment of solid tumors) at nanomole per liter concentrations that inhibited downstream constitutive IGF1R phosphorylation. In addition, QGP-1 proliferation was sensitive to chemotherapeutic 17-AAG (an Hsp90 small molecule inhibitor in phase II clinical trials for treatment of advanced malignancies) at nanomole per liter concentrations that induced loss of client proteins EGFR, IGF1R, and VEGFR2. Finally, the therapeutic agent axitinib (a TKI targeting VEGFR1-3/PDGFR-A/B/KIT in phase II clinical trials for treatment of solid tumors) inhibited QGP-1 growth at nanomole per liter concentrations that increased expression of cyclin-dependent kinase inhibitor p21 without inhibiting constitutive VEGFR2 phosphorylation, a pattern suggestive of treatment-induced senescence in cancer (review Ewal et al<sup>40</sup>).

Although this report is the first immunohistochemical study of IGF1R in a heterogeneous collection of PETs, IGF1R was detected by IHC in 31 (of 32) gastrinomas assessed previously.<sup>41</sup> No clinical trials of anti-IGF1R small molecule TKI monotherapy for PETs have been undertaken, for comparison of treatment response to data presented here on in vitro BMS-754807 antiproliferative activity and strong PET IGF1R expression. However, anti-IGF1R monoclonal antibody monotherapy for PETs has been investigated in clinical studies. Results from a recent phase II clinical trial with dalotuzumab (MK-0646) indicated an absence of antitumor activity in 10 patients with metastatic PETs; IGF1R tumor expression data were unavailable.<sup>42</sup> Of note, it is well established that functional reciprocal cross-talk between EGFR and IGF1R occurs in other forms of cancer,<sup>43</sup> in which adaptive activation of HER family members occurs upon inhibition of IGF1R (and vice versa). Thus, further research into developing anti-IGF1R TKIs for potential PET anticancer treatment might benefit from investigating these therapeutics as part of multidrug, rather than single-agent, strategies targeting the HER family of receptors as well.

Similarly, no clinical studies in patients with PETs have been performed with single-drug axitinib, the more selective and potent of the 2 anti-VEGFR1-3/PDGFR-B TKIs analyzed in QGP-1 cells, for comparison of treatment response to data here on in vitro antiproliferative activity and strong tumor expression of VEGFR1 and PDGFR. However, sunitinib monotherapy has been investigated in phase II and III clinical trials in patients with PETs, although no VEGFR1-3 and PDGFR-B tumor expression data are available.<sup>6,44</sup> Characteristics of the PET growth inhibition induced by axitinib and sunitinib in vitro and in vivo, respectively, are consistent with a cytostatic mechanism. Axitinib induced evidence of senescence in PET cells, a response possibly exploitable in follow-up combination studies with conventional chemotherapeutics cytotoxic to growth arrested cells. Sunitinib monotherapy in a phase II trial in patients with PETs induced a level of tumor shrinkage classified as stable disease by Response Evaluation Criteria in Solid Tumors in most patients (62.1%), whereas the objective response rate was 16.7%.<sup>44</sup> Furthermore, in a recent phase III study of sunitinib monotherapy in patients with PETs, the median progression-free survival in the sunitinib-treated group was more than double that of the placebo-treated group, whereas the objective response rate was 9.3%.<sup>6</sup> Taken together, these results are encouraging of combination, rather than monotherapy, studies of axitinib to fully explore its potential for development as antitumor therapy in PETs, that is, by combining axitinib with conventional chemotherapeutics toxic to tumor cells in growth arrest.

In contrast to the in vitro effects of TKIs BMS-754807 and axitinib, antiproliferative concentrations of inhibitor 17-AAG, targeting the ubiquitous molecular chaperone Hsp90, depleted multiple growth factor receptors in QGP-1 cells in a concurrent fashion (EGFR, IGF1R, and VEGFR2). No clinical trials of 17-AAG monotherapy for PETs have been undertaken; however, Hsp90 was moderately strongly expressed in most of these tumors, suggesting that further investigation into the potential for targeting Hsp90 for development of new anticancer drugs for this malignancy might benefit from multidrug strategies that include a cytotoxic chemotherapeutic with a different mode of action.

Pancreatic endocrine tumors are commonly diagnosed at advanced stages of disease for which few current therapies induce high tumor regression rates. Ongoing investigations in our laboratory are based on this multifaceted study, which encouraged further research into the role in some PETs of multidrug strategies incorporating anti-EGFR monoclonal antibody,

anti-IGF1R TKI BMS-754807, or Hsp90 inhibitor 17-AAG as well as combination studies with axitinib and conventional chemotherapeutics maximally toxic to growth-arrested cancer cells.

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