Molecular Targets in Human Neuroendocrine Tumors

Ujalla Sheikh, Jalil Muhammad, Domenico Coppola, and Aejaz Nasir

Introduction

Recent data demonstrate that the incidence of gastroenteropancreatic neuroendocrine tumors (GEP-NETs) has increased exponentially (overall ~500 %) over the last three decades, thus refuting the erroneous concept of their rarity [1, 2], due in part to improved diagnostic services and greater public awareness. Diagnosis of these neoplasms is usually delayed since there is no biochemical screening test and symptoms are protean and overlooked. Although grouped as a neoplastic entity (NETs), each lesion is derived from distinct cell precursors, produces specific bioactive products, exhibits distinct chromosomal abnormalities and somatic mutation events, and has uniquely dissimilar clinical presentations. GEP-NETs demonstrate very different survival rates reflecting the intrinsic differences in malignant potential and variations in proliferative regulation [1]. Specifically for PNETs, the clinical course depends primarily on the type of primary tumor, the tumor size, and histological grade [3]. Functional tumors may present at an early stage due to hormonal symptoms arising from secretion of various hormones or amines by the tumor [3]. The vast majority of PNETs, however, are nonfunctional, tend to remain clinically silent and may result in larger tumor size at presentation, compared to their functional counterparts. About two-thirds of patients with pancreatic NETs have distant metastases at diagnosis [2].

U. Sheikh • D. Coppola, MD
Department of Anatomic Pathology, H. Lee Moffitt Cancer Center, Tampa, FL, USA

J. Muhammad
Department of Neuroscience, George Mason University, Fairfax, VA, USA

A. Nasir, MD, MPhil, FCAP (✉)
Diagnostic and Experimental Pathology (Tailored Therapeutics), Lilly Research Laboratories, Eli Lilly and Co., Room 2391, Building 98C/2C, 893 South Delaware St., Indianapolis, IN, USA
e-mail: nasirae@lilly.com

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In recent years, there has been a dramatic increase in the number of molecularly targeted agents to treat cancer as a result of improved understanding of the complex pathobiologic processes and molecular pathways that are involved in the development, growth and progression of cancer, including NETs. Here we would like to review some of the key molecular targets that have been investigated as potential therapeutic targets in pancreatic NETs in recent years and review some of the emerging data, which may be of relevance to the diagnostic pathologists who are actively engaged in multidisciplinary care of patients with GEP/pancreatic neuroendocrine tumors. The molecular targets that have been under active clinical and scientific investigation in recent years [4] include peptide hormone receptors and receptor tyrosine kinases, including those that have a well-defined role in pathologic angiogenesis in PNETs and intracellular molecular targets like the mammalian target of rapamycin (mTOR).

Peptide Receptors and NETs

For many years somatostatin analogs (SSAs) have been used as a form of targeted therapies to control symptoms of hormonal hypersecretion by functional NETs. These agents are still an important treatment modality for NETs. In recent years somatostatin receptors (SSTRs) have been under active investigation as important targets for diagnosis and treatment of NETs.

Somatostatin and Somatostatin Receptors (SSTRs)

Somatostatin is an endogenous cyclic peptide that regulates the secretion of growth hormone, insulin, glucagon, and gastrin by the respective endocrine cells [5], through a family of G protein-coupled transmembrane receptors, including five distinct subtypes (SSTRs 1–5) [6, 7]. Somatostatin has a very short half-life (~3–4 min) [7], limiting its therapeutic efficacy in clinical setting. However, synthetic somatostatin analogs (SSAs) such as octreotide and lanreotide have high affinity for SSTR2 and SSTR5 and are without the undesirable effects of somatostatin [7, 8], supporting their clinical usefulness.

Activation of SSTRs has a number of direct and indirect effects on NET cells [9]. Direct antiproliferative effects of SSTR activation include inhibition of cell cycle and growth factor effects and induction of apoptosis, which may be mediated by the PI3K/mTOR, MAPK, and Ras/ERK signaling pathways [10, 11]. Indirect effects of SSTR activation include inhibition of the release of growth factors and trophic hormones, inhibition of angiogenesis, and modulation of the immune system [9].

Because of clinical and biologic relevance of SSTRs, a number of studies have investigated the expression and distribution of SSTRs in archival human NET tissues [12–14]. The distribution of SSTRs is widespread in GEP-NETs, with an overall prevalence of 50–100 % and frequent co-expression of multiple SSTRs in a given tumor. The expression of SSTRs varies among various histological types of

NETs and also among patients with the same tumor type. SSTR2 and SSTR5 are expressed in about 90 % and 80 % of pancreatic NET cells, respectively, making them potentially sensitive to hormone treatment [15]. Somatostatin receptor 2 (SSTR2) was absent or very low in insulinomas compared with nonfunctioning PNETs; differential expression of various SSTRs in PNETs makes evaluation of SSTRs relevant as markers of response to somatostatin analogs [16].

Other Peptide Receptors

With the clinical success of SSTRs in the management of NETs, patients have led to increased interest in other peptide receptors, including receptors for bombesin, cholecystokinin, and vasoactive intestinal peptide (VIP) in some types of pancreatic NETs [17–19].

Epidermal Growth Factor Receptor (EGFR)

Epidermal growth factor family of receptors (EGFR, ErbB) consists of four structurally related receptor tyrosine kinases (RTKs), including HER1 (aka EGFR, ErbB1), HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4). Eleven growth factor ligands can activate EGFR family of receptors, including EGF and TGF-alpha. EGFR (ErbB-1) is one of the regulator of the PI3K/Akt and the MAPKs pathways, which are important in regulating a number of cell functions, including cell growth, proliferation, differentiation, motility, and survival. EGFR activation pathways have been well characterized using tumor cell lines and are known to involve EGFR activation through autophosphorylation [20]. EGFR activation results in phosphorylation/upregulation of downstream signaling molecules, such as ERK1/2 (extracellular regulated kinase 1 and 2) and PKB/Akt (protein kinase B), which leads to enhanced tumor cell survival and proliferation [20]. Regulation of EGFR activity can be disrupted by several mechanisms: increased production of ligands; overexpression of EGFR; impaired downregulation of EGFR; cross-talk between EGFR and other EGFR family members, TKIs, and receptors; and activation of mutations in EGFR.

EGFR in NET Cell Lines

In a mutational survey of 36 kinase genes, including RTKs (EGFR, c-Kit, HER2, PDGFR-alpha), 6 genes from the Akt/mTOR pathway (AKT2, PIK3CA, RPS6K1, STK11, PDPK1, FRAP1-mTOR), and 25 genes that are frequently mutated in cancer, revealed alterations in targetable kinases in neuroendocrine cancer cell lines. PNET cell lines QGP1, CM, and BON harbored mutations in FGFR3, FLT1/VEGFR1, and PIK3CA, respectively, rendering these models useful for preclinical studies involving pathway-specific therapies [21].

In a recent study the effect of transactivation of EGFR by EGF, TGF-alpha, and various GI hormones to stimulate the growth of human foregut carcinoid (BON), the somatostatinoma (QGP-1), and the rat islet tumor (Rin-14B) cell lines showed an increased Tyr(1068) EGFR phosphorylation [22]. Furthermore, the stimulated phosphorylation of EGFR was dependent on Src kinases, PKCs, MMPs, and reactive oxygen species. These findings suggest that disruption of EGFR signaling cascade by EGFR inhibition alone or combined with other receptor antagonists may be a novel therapeutic approach for treatment of foregut NETs and PETs [22].

Cell Lines with Mutations in Other Tyrosine Kinase Genes

PNET cell lines QGP1, CM, and BON have been shown to harbor mutations in FGFR3, FLT1/VEGFR1, and PIK3CA genes, respectively [21]. These findings may have relevance to design preclinical studies with respective targeted therapies.

EGFR in Human NETs/PNETs

Several immunohistochemical studies have reported EGFR expression in human pancreatic NETs [23], with the proportion of samples expressing EGFR ranging from 18 to 65 %, including variation in the intensity of EGFR staining. The observed variation among studies may reflect differences in the patient populations or antibodies used [24] and need careful evaluation of this important therapeutic target in larger cohorts of pancreatic and non-pancreatic NETs.

Differential Expression of EGFR in Human GI and Pancreatic NETs

In an IHC-based analysis of 140 human PNETs, EGFR was immunopositive in 18 (13 %), HER2 in 3 (2 %), KIT in 16 (11 %), and PDGFR-alpha in 135 (96 %) [21]. These RTKs were expressed in PNET cells with variable frequency and/or in the surrounding tumor endothelium (Fig. 1).

Of the 130 PNETs evaluated as a tissue microarray (TMA) by FISH, 2 (1.5 %) cases had HER2 gene amplification [21]. All of these PNETs were disomic, monosomic (Fig. 2), or polysomic-trisomic, but there was no EGFR gene amplification [21].

An IHC, Western blot, and qPCR-based analysis of EGFR and P-EGFR expression in GI carcinoids and PETs showed a higher percentage of primary and metastatic GI carcinoids expressed EGFR and P-EGFR compared to PNETs. However, PNET patients with activated EGFR had worse prognosis [24]. These findings implicate the EGFR and P-EGFR signal transduction pathway in the pathogenesis of these tumors and suggest that targeted therapy directed against the EGFR tyrosine kinase domain may be a useful therapeutic approach in patients with unresectable metastatic GI carcinoids and PNETs [24].

In another IHC-based investigation of human PNETs, 96 % of tumor samples were *positive* for EGFR expression, 63 % for activated EGFR, 76 % for activated

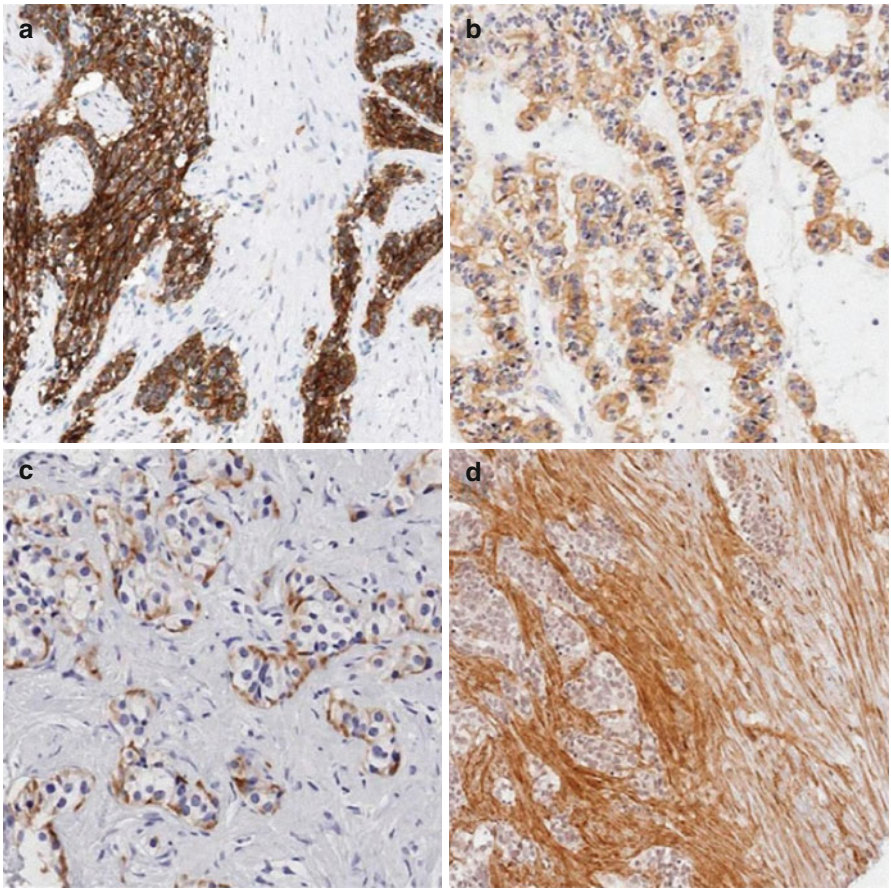

Fig. 1 Immunohistochemical staining for receptor tyrosine kinases in pancreatic endocrine tumors. Shown are positive staining for KIT (**a**), EGFR (**b**), and HER2 (**c**) in tumor cells; positive staining in tumor cells and in the stroma is shown for PDGFR-alpha (**d**). Original magnification, ×20 (Reproduced with permission from Corbo et al. [21])

Akt, and 96 % for activated ERK1/2 [20]. Furthermore, the histological score for the activation of Akt and ERK1/2 correlated with the histological score for activated EGFR. Based on these findings, the investigators suggested that therapeutic inhibition of EGFR with or without concomitant inhibition of Akt and ERK1/2 may provide newer therapeutic options for NET patients [20].

EGFR as a Prognostic Factor in Pancreatic NETs

Co-expression of transforming growth factor-alpha (TGF-alpha) and its receptor epidermal growth factor receptor (EGFR) is known to be associated with aggressive biologic behavior and adverse clinical outcome in a variety of tumors, including pancreatic adenocarcinomas [25]. Although expression of EGFR has been disputed as a

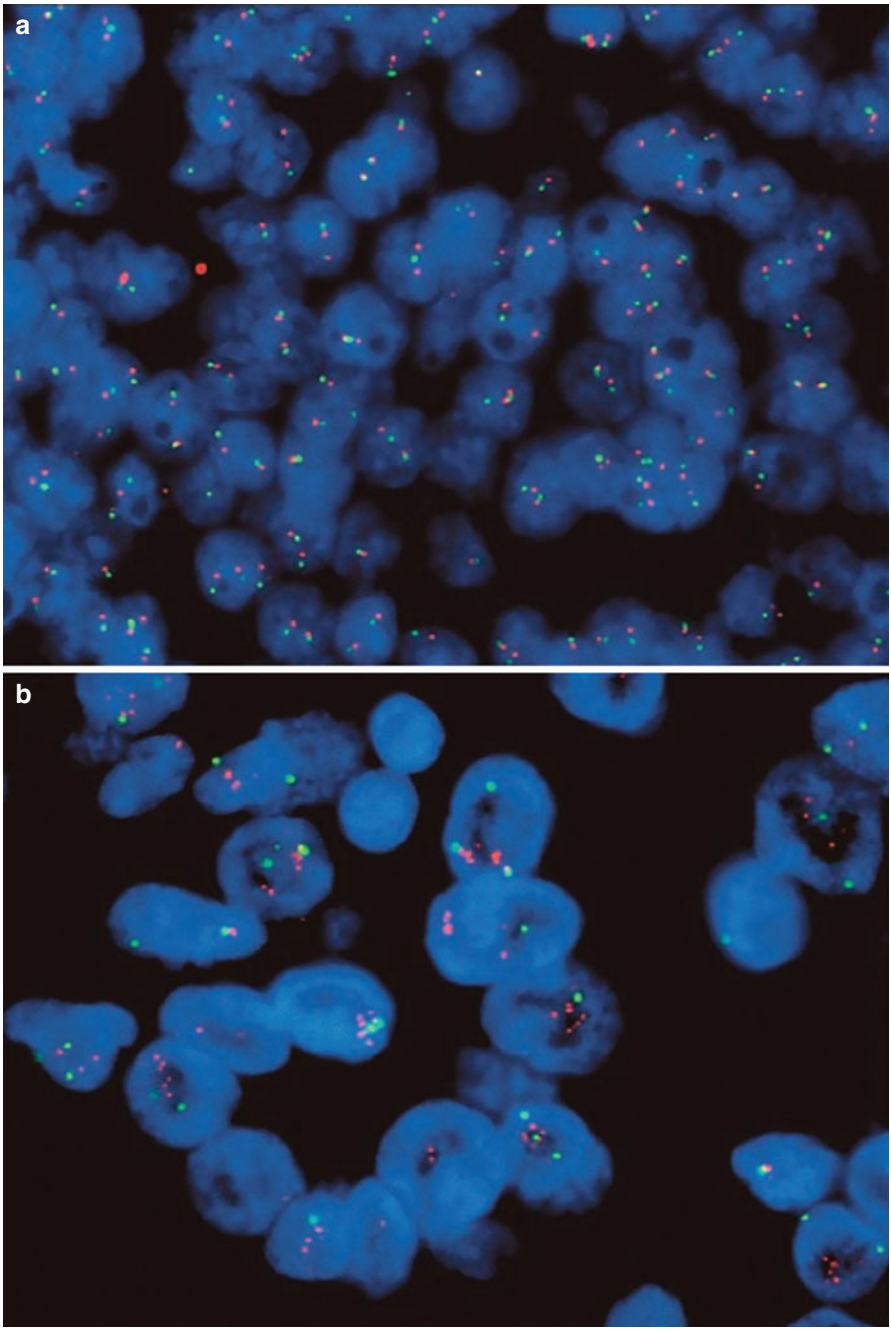

Fig. 2 Fluorescent in situ hybridization (FISH) analysis for EGFR and HER2 in pancreatic endocrine tumor. FISH analysis showing monosomy for EGFR (**a**) and gene amplification for HER2 (**b**). Originalmagnification,·100. EGFR and HER2 signal red, centromeric probes signal green (Reproduced with permission from Corbo et al. [21])

marker of malignancy in NETs [25, 26], a recent study showed significant correlation between EGFR expression and grade of malignancy in pancreatic NETs with low levels of expression in benign tumors and those of uncertain behavior to high levels of expression in well and poorly differentiated tumors [27]. Furthermore, patients with pancreatic NET expressing activated EGFR have been found to have significantly worse prognosis than those whose tumors did not express activated EGFR [24].

Collectively, findings in the above studies suggest that EGFR may provide useful information both as a prognostic marker in GEP-NETs. Furthermore, targeted therapy against the tyrosine kinase domain of EGFR may be a clinically relevant approach for patients with GI and pancreatic NETs.

Stem Cell Factor Receptor (SCFR aka c-Kit, Tyrosine Protein Kinase, CD117)

c-Kit is a protein that in humans is encoded by the gene, KIT. KIT is expressed on the surface of hematopoietic stem cells and other cell types. Receptor is activated by binding with its ligand, stem cell factor (SCF, or KIT-ligand). A number of studies have evaluated expression of c-Kit in human pancreatic NET tissue samples [28–31]. The proportion of c-Kit positive samples varied over a wide range between these studies and within one study varied substantially with the type of antibody used to detect c-Kit [29]. Inconsistencies between studies may, therefore, be related to technique or antibodies used rather than c-Kit levels. It is, therefore, important to define the most optimal approach to assess the KIT status of a given PNET and to determine if c-Kit expression by NETs can be translated into therapeutic benefit with agents like imatinib mesylate [29]. More recently, in a multivariate analysis of several prognostic factors, only WHO criteria and c-Kit expression were identified as independent markers of unfavorable prognosis in pancreatic NETs [32]. Furthermore, based on IHC expression of KIT and CK19 expression, PNETs were grouped into three prognostic categories: low risk (KIT−/CK19−), intermediate risk (KIT−/CK19+), and high risk (KIT+/CK19+), with significantly different patient survival, metastases, and recurrence of PETs among the three groups.

Sarcoma Kinase (Src)

The Src family of kinases (SFK) is a family of non-receptor tyrosine kinases involved in the transduction of signals from the cell membrane to different targets involved in cell cycle, cell adhesion, and cell motility. SFK activity has been shown to regulate adhesion, spreading, and migration of pancreatic NET cells in vitro [33]. Similarly, SFKs were found to be overexpressed in human PNETs [34] and are also involved in the transactivation of EGFR [22]. Inhibition of Src activity decreases adherence, spreading, and migration of pancreatic NET cells in vitro [33]. Furthermore, SFKs

regulate mTOR activity during adhesion and simultaneous inhibition of SFKs and mTOR reduces proliferation of PNET cells without inducing PI3K/Akt activity [33].

Intracellular and Downstream Targets

Intracellular molecules mediating signal transduction downstream of RTKs form the basis of additional therapeutic approaches against pancreatic NETs. One of these targets is mTOR – a serine/threonine kinase, which regulates cell growth, metabolism, and apoptosis.

mTOR Pathway

mTOR is one of the most important target among a large number of extracellular and intracellular signaling molecules. Being a key regulator of several different cell functions, mTOR activation is tightly controlled by several positive and feedback regulatory loops [35]. Furthermore, mTOR forms two distinct protein complexes (mTORC1, mTORC2) (Fig. 3), which can be activated in different ways and exert different but related functions [37]. Mutations in the mTOR pathway genes have been reported in 15 % of PNETs [38]. Among these, the most important regulatory genes include phosphatase and tensin homolog (PTEN) [39], the tuberous sclerosis complex 2 gene (TSC2) [40, 41], and neurofibromatosis type 1 (NF1) [42, 43]. Loss-of-function mutations in TSC1 and TSC2, tumor suppressor genes that inhibit mTOR, occur in tuberous sclerosis – a hereditary cancer syndrome associated with the development of PNETs [44]. PTEN regulates the activity of mTOR through the Akt pathway and, along with TSC2, is downregulated in approximately 75 % of the primary PNETs, supporting a role for the PI3K/Akt/mTOR pathway in the development of pancreatic NETs. Also, low expression of these molecules was associated with shorter disease-free and overall survival in primary PNETs [16].

Clinical Success with mTOR Inhibitors and Future Clinical Opportunities

The mTOR inhibitor everolimus, which specifically inhibits mTORC1 and also mTORC2 (on prolonged exposure to drug), showed activity in a phase II study, followed by randomized, placebo-controlled phase III trials in patients with advanced PNETs and extra-pancreatic carcinoids. Based on significant increase in PFS in patients, receiving everolimus (vs. placebo) led to its approval [45]. One of the major clinical challenge is posed by the concomitant activation of the PI3K and MAPK pathways [46–48], which may interfere with the activity of mTOR inhibitors and may need alternative therapeutic approaches, like using mTOR inhibitors with or without SSTR analogs or as dual PI3K/mTOR inhibitors in advanced NETs

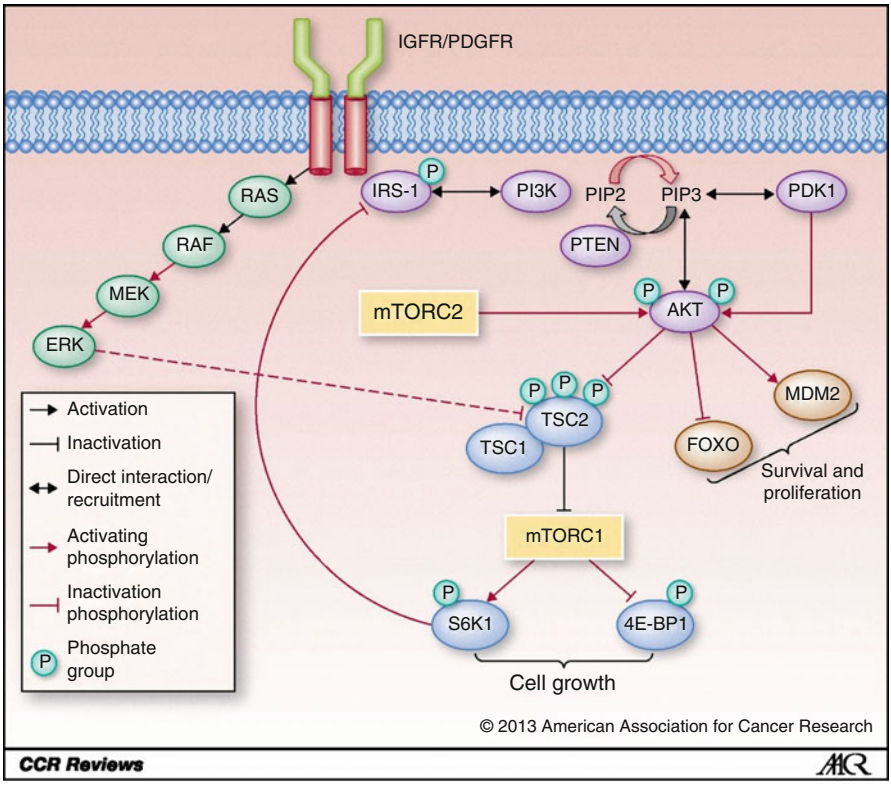

Fig. 3 Schematic representation of the mTOR pathway and associated regulatory circuitries. mTOR exists as two different complexes (mTORC1 and mTORC2) that are activated through different signaling cascades. Here the activation of mTORC1 by receptor tyrosine kinases–triggered signaling is depicted. Positive and feedback regulatory loops are also described. *PIP2* phosphatidylinositol (4,5)-bisphosphate, *ERK* extracellular signal-regulated kinase, *IGFR* insulin-like growth factor receptor, *MEK* MAP-ERK kinase, *PDGFR* platelet-derived growth factor receptor, *PI3K* phosphoinositide 3-kinase, *PIP2* phosphatidylinositol (4,5)-bisphosphate, *PIP3* phosphatidylinositol (3,4,5)-trisphosphate (Reproduced with permission from Oberg et al. [36])

(pancreatic and extra-pancreatic) [49, 50]. The rationale for combination of mTOR inhibition with SSTR analogs is to inhibit the IGF1R/PI3K/Akt axis (Bousquet, 2006 #167).

Other Potentially "Druggable" Targets

Several other potential therapeutic targets have been identified in human pancreatic NETs, including IGF-1, B-Raf [51], COX-2 [52], MTA-1 [53], CDK4 [54], claudin 3 and 7 [55], MAGE1 [56], and activated Akt [57]. Clearly there is need to develop

and standardize methodologies to evaluate these targets at protein and RNA/DNA levels in well-characterized human GEP-NET tissues.

Dopamine Receptors

Dopaminergic drugs have been proposed to have an antiproliferative effect in functional pancreatic NETs [58]. A number of studies have investigated dopamine 2 receptor expression in NETs [59–62]. Grossrubatscher et al. [60] showed high expression of these receptors in 85 % of NETs (mostly pancreatic and lung). Furthermore, dopamine 2 receptor immunoreactivity was present in 93 % of the islet cell tumors studied. Generally, high positivity was reported in more than 70 % of tumor cells, particularly in bronchial and pancreatic tumors. The authors conclude that there may be a role for dopaminergic drugs in inhibiting secretion and/or cell proliferation in NETs.

Co-expression of Dopamine 2 and Somatostatin Receptors in NETs

Co-expression of dopamine 2 receptors with SSTR2 and SSTR5 has also been reported, with higher expression of the dopamine receptors in low-grade rather than high-grade NET [63]. Kidd et al. [64] report variable expression of dopamine 2 and somatostatin receptors depending on cell type and tissue of origin. In future studies, it will be valuable to develop methodologies that can reliable quantify both of these two receptors in archival human NET tissues.

Abbreviations

Akt	Protein kinase B
AKT2	kt/mTOR pathway gene
ALDH+	Aldehyde dehydrogenase positive
ATM	Protein kinase
BON	Pancreatic neuroendocrine tumors cell line
c-Kit	Stem cell growth factor receptor
CK19	Cytokeratin 19
CM	Pancreatic neuroendocrine tumors cell line
CSC	Cancer stem cells
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ErbB-1	Epidermal growth factor receptor
ERK	Extracellular-regulated kinase
ERK1	Extracellular-regulated kinase 1

ERK2	Extracellular-regulated kinase 2
FGFR3	Fibroblast growth factor receptor 3
FLT1	EGFR gene
FRAP1-mTOR	Akt/mTOR pathway gene
GEP-NET	Gastroenteropancreatic neuroendocrine tumor
GI	Gastrointestinal
HER	Human epidermal growth factor receptor
HER2	Human epidermal growth factor receptor 2
HER3	Human epidermal growth factor receptor 3
IHC	Immunohistochemistry/immunohistochemical
KIT	c-Kit encoding gene
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinases
mTOR	Mammalian target of rapamycin
mTOR	Mammalian target of rapamycin
NET	Neuroendocrine tumor
NF1	Neurofibromatosis type 1
PDGFR-alpha	Platelet-derived growth factor receptor-alpha
PDPK1	Akt/mTOR pathway gene
P-EGFR	Phosphorylated EGFR
PIK3CA	Akt/mTOR pathway gene
PI3K	Phosphoinositide-3-kinase
PKB	Protein kinase B
PMET	Phosphorylated MET
PNET	Primitive neuroendocrine tumor
PTEN	Phosphatase and tensin homolog
QGP-1	Pancreatic neuroendocrine tumors cell line
qPCR	Quantitative polymerase chain reaction
Rin-14B	Pancreatic delta cell line
RPS6K1	Akt/mTOR pathway gene
RTK	Receptor tyrosine kinase
SCF	Stem cell factor
SFK	Sarcoma family of kinases
siRNA	Small interfering RNA
Src	Sarcoma
SSA	Somatostatin analog
SSTR	Somatostatin receptors
SSTR2	Somatostatin receptor 2
SSTR5	Somatostatin receptor 5
STK11	Akt/mTOR pathway gene
TGFα	Transforming growth factor-alpha
TSC1	Tuberous sclerosis complex 1 gene
TSC2	Tuberous sclerosis complex 2 gene
VEGFR1	VEGF receptor
VIP	Vasoactive intestinal peptide

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