Molecular Pathogenesis of Neuroendocrine Tumors: Implications for Current and Future Therapeutic Approaches

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Abstract

The treatment landscape and biological understanding of neuroendocrine tumors (NETs) has shifted dramatically in recent years. Recent studies have shown that somatostatin analogs have the potential to not only control symptoms of hormone hypersecretion, but also have the ability to slow tumor growth in patients with advanced carcinoid. The results of clinical trials have further shown that the vascular endothelial growth factor (VEGF) pathway inhibitor sunitinib and the mammalian target of rapamycin (mTOR) inhibitor everolimus have efficacy in patients with advanced pancreatic NETs. The efficacy of these targeted therapies in NET suggests that the molecular characterization of NETs may provide an avenue to both predict which patients may most benefit from treatment and to overcome potential drug resistance. Recent genomic studies of NETs have further suggested that pathways regulating chromatin remodeling and epigenetic modification may play a key role in regulating NET growth. These observations offer the potential for new therapeutic and diagnostic advances for patients with NET.
Introduction

Analysis of the Surveillance, Epidemiology, and End Results (SEER) database has suggested an increasing incidence of neuroendocrine tumors (NETs) from 1.09 cases per 100,000 in 1973 to 5.25 per 100,000 in 2004 (1). While several advances in the treatment of NETs have been made in recent years, additional therapeutic options are needed.

Molecular Pathways

Angiogenesis

NETs are vascular tumors exhibiting a high expression of several pro-angiogenic molecules such as the angiogenic cytokine vascular endothelial growth factor (VEGF) (2). Several VEGF pathway inhibitors, including the VEGF inhibitor bevacizumab and the VEGFR-targeted tyrosine kinase inhibitors sunitinib, pazopanib, and sorafenib have demonstrated clinical activity in NET. In 2011, sunitinib was evaluated in a randomized, placebo-controlled clinical trial of pancreatic NET (PanNET) and shown to more than double progression-free survival, leading to its approval for this indication by the Food & Drug Administration (FDA) and the European Medicines Agency (EMEA). These data form the rationale for the use of anti-angiogenic therapies in NET (3). Despite these advances, some tumors demonstrate intrinsic resistance to anti-angiogenic therapies, whilst acquired resistance develops in others.

Both intrinsic resistance and acquired resistance share similar molecular and cellular mechanisms (Figure 1). With both mechanisms, resistance is caused by the expression of multiple pro-angiogenic factors including VEGFs, fibroblast growth...
factors (FGFs), angiopoietins, and ephrins, which can overcome single-agent VEGF-VEGF receptor targeted therapy (4, 5). Several mechanisms have been postulated to be inducers of upregulation of these pro-angiogenic factors, including intra-tumor hypoxia with HIF1α accumulation, inducing a hypoxia-stress expression program that includes many pro-angiogenic factors (6, 7). Amongst the genes directly regulated by HIF-1, c-Met is involved in the invasive and metastatic behavior of tumor cells after the exposure to hypoxia. Recent studies have demonstrated that inhibition of c-Met can reduce the invasive and metastatic capabilities promoted following VEGF-pathway inhibition, suggesting that inhibition of c-Met, together with VEGF-targeted therapy, may diminish resistance (8).

Additional strategies to overcome these resistance mechanisms have been explored in animal models and in the clinical setting (9). For example, studies in xenograft models have demonstrated that the combination of bevacizumab and HIF-1 or Sp1 inhibitors may increase the therapeutic efficacy of anti-angiogenic treatment (10, 11). Co-targeting of VEGF and FGF signaling pathways has also been shown to improve efficacy and overcome adaptive resistance to VEGF inhibition in the RIP-Tag2 model of PanNETs (12).

**mTOR pathway**

Mammalian target of rapamycin (mTOR) is the convergence hub of a variety of extracellular and intracellular signals. As a master regulator of different cell functions, mTOR activation is subjected to tight and coordinated regulations through diverse positive and feedback regulatory loops (Figure 2) (13). Moreover, mTOR forms two distinct protein complexes, commonly referred as mTORC1 and mTORC2, which are activated in different ways and exert different but related functions (14). Mutations in
the mTOR pathway have been reported in 15% of PanNETs (15). Loss of function mutations in TSC1 and TSC2, tumor suppressor genes that inhibit mTOR occur in tuberous sclerosis, a hereditary cancer syndrome that is associated with the development of PanNET (16). Phosphatase and tensin homolog (PTEN), which regulates the activity of mTOR through the Akt pathway, together with TSC2 are downregulated in approximately 75% of PanNETs and their low expression is associated with shorter disease-free and overall survival (17).

Following evidence in phase II studies that mTOR inhibitors had activity in NETs, the mTOR inhibitor everolimus was evaluated in randomized, placebo-controlled trials enrolling patients with advanced PanNET or other advanced extrapancreatic NET (carcinoid). The phase III study of everolimus enrolled 410 PanNET patients and demonstrated a significant increase in progression free survival (PFS) in patients receiving everolimus as compared with those receiving placebo (11 vs. 4.6 months), leading to its approval by the FDA and EMEA for this indication (18). A parallel study in patients with advanced extrapancreatic NET/carcinoid also suggested that everolimus might have some activity, although the study did not meet its pre-defined efficacy endpoint (19).

Everolimus is known to specifically inhibit mTORC1, although prolonged drugs exposure might impair mTORC2 activity in a cell-type specific manner. The efficacy of everolimus and other rapamycin analogs may be compromised by feedback loop mechanisms that include the concomitant activation of PI3K and MAPK pathway (Figure 2) (20–22). Strategies to counteract this feedback loop have included the development of ATP-competitive mTOR inhibitors targeting both mTOR complexes. In vitro and in vivo data suggest that these new compounds have therapeutic benefit over rapamycin, but concerns exist about their potential toxicity.
An alternative approach is the use of dual PI3K/mTOR inhibitors for which several clinical trials are underway (23). Potential clinical benefit has also been shown in patients with PanNET who have been treated with a combination of everolimus and somatostatin (SST) analogs (24). One rationale for this combination relies on the known effect of SST analogs in dampening the IGFR/PI3K/Akt axis (25). Strategies using dual mTOR inhibitors targeting mTORC1 and mTORC2, combining mTOR inhibition and inhibition of PI3K, and combining mTOR inhibitors with SST analogs are thus of significant interest.

**Somatostatin receptor signaling**

SST and its synthetic analogues (e.g. lanreotide, octreotide) act through a family of five G-protein couple receptors termed sst1-sst5 to exert a variety of functions, including inhibition of endocrine and exocrine secretions and of tumoral cell growth (26, 27).

While SST analogs are associated with high symptomatic response rates initially, patients may develop resistance to treatment over time (28–30). The molecular mechanisms underlying the development of resistance remain poorly understood. However, recent studies have identified a novel truncated sst5 receptor variants in rodents (31, 32) and humans (33), which at variance with full-length canonical sst5, display selective responses to SST and cortistatin, and exhibit distinct tissue distribution and a unique subcellular localization. One variant, sst5TMD4, which is barely expressed in normal human tissues, shows a marked upregulation in tumors, where it appears to entail pathologically relevant functions. Thus, for example, expression of sst5TMD4 in pituitary adenomas causing acromegaly is related to the reduced ability of octreotide at normalizing hormone
secretion in poorly responsive tumors *in vivo* (34). In breast cancer, the presence of sst5TMD4 (which is negligible in normal mammary gland) is associated with markers of poor prognosis (lower levels of ER, HER2/Neu and p53) and its expression in breast cancer model cell lines was shown to increase malignancy features (cell proliferation, signaling, invasiveness, and migration) by disrupting normal sst2 function (35). Such observations suggest a potential role for these novel truncated receptor variants in tumor diagnosis as a prognostic marker and as therapeutic targets.

**Target Discovery in Neuroendocrine Tumors**

*Sequencing efforts in NET*

The recognition of driver oncogene mutations in a number of human cancers has provided a compelling rationale for targeted therapies (Table 1). Recently, full exome sequencing of ~18,000 genes in sporadic well-differentiated PanNETs revealed 157 mutations in 149 genes. The most common of these were multiple endocrine neoplasia type 1 (*MEN1*), death domain associated protein (*DAXX*), *ATRX*, *PTEN*, and *TSC2* (15). A *MEN1* mutation was carried by 44% of tumors and 43% had either a *DAXX* or *ATRX* mutation.

Mutations have been identified at multiple sites in the *MEN1* gene (36). The majority of PanNETs show qualitative and/or quantitative alterations in menin localization (36). In 30% of cases, this was associated with *MEN1* mutations affecting sequences involved in nuclear localization or protein–protein interaction. Details of the crystal structure of menin have recently been published and provide structure-function explanations for the distinct interactions with the MLL and JUND
proteins (37). These insights offer the potential for molecular targeting and specific drug development.

While the MEN1 gene has been well studied, the functions of DAXX and ATRX are less well understood. DAXX and ATRX proteins form a complex that localizes to chromatin and PML bodies (38). DAXX is a histone H3.3 chaperone and the ATRX-DAXX complex assembles H3.3 into nucleosomes. ATRX can recruit DAXX to deposit H3.3 at telomeres or pericentromeric heterochromatin. How chromatin remodeling mediated by ATRX and DAXX contributes to NET pathogenesis remains to be defined, but one potential mechanism is the regulation of telomeres. Alternative lengthening of telomeres occurs through DNA recombination and has been shown to be positive in 61% (25/41) of PanNETs; 19 of these tumors had mutations in ATRX or DAXX (39). Further investigations on how chromosome structure alters and the consequence of the switched ‘on’ and ‘off’ genes when cells lack ATRX-DAXX may facilitate the identification of novel molecular targets for future diagnostic modality and therapeutic intervention in PanNETs.

Protein kinases

Protein kinases represent another potential therapeutic target in NET. Of 356 kinase genes studied in the sequencing project, three mutations in primary PanNET were identified in the ATM and KIT genes (40). In cell lines, mutations in the following genes were also identified: FGFR3, FLT1 and PI3KCA. KIT membrane expression is associated with shorter patient survival in patients with PanNETs (40).

An additional potential target for the treatment of NETs are the Src Family Kinases (SFKs). Members of the SFKs are overexpressed in PanNETs (41) and
inhibition of their activity impairs adhesion, spreading and migration of PanNET cells (42). A novel role for SFKs in controlling mTOR activity in PanNET cells has also been reported (43). The increased mTOR activity controlled by SFK leads to translation of a subset of mRNAs for cell cycle progression. Moreover, the concomitant inhibition of SFK and mTOR activities strongly impaired cell growth, compared with the effect exerted by the single agents. Notably, while treatment with mTOR inhibitors triggered the activation of a prosurvival feedback dependent on PI3K/Akt signaling, the simultaneous inhibition of SFKs blocked this escape signal. These results and the recent findings of an important role of the Src pathway in modulating the growth of neuroendocrine cancer stem cells in vitro, and in vivo (44), support the need for further preclinical studies with SFK inhibitors.

**Epigenetic characterization of NETs**

Epigenetic mechanisms are an essential component of normal development and gene expression patterns in mammals. Disruption of such processes can result in altered gene function and malignancy through changes in DNA methylation, histone modifications, inappropriate nucleosome positioning and non-coding RNAs, specifically microRNA (MiRNAs) expression (45). Mutations in epigenetic regulators have the potential to lead to misregulation of gene expression that contributes to tumorigenesis and it has been suggested that epigenetic rather than genetic changes may play a key role in NETs (46).

The longstanding observation that menin mutations are associated with NETs supports a role for epigenetic regulation in the pathogenesis of this disease. Menin is part of a histone methyltransferase complex; it associates with p27^{Kip1} and p16^{ink4c} promoters to methylate histone H3 and enhance their transcription. Menin deficiency
results in downregulation of p27^Kip1 and p18^INK4c (47, 48). Histone H3 methylation is reduced in islet tumors from MEN1 mutant mice. In addition, mice deficient for both p27^Kip1 and p18^INK4c develop pituitary tumors much more rapidly than either deficiency alone, suggesting the two inhibitors collaborate to suppress tumorigenesis (49). Collectively, these observations point to a key role for menin in epigenetic regulation.

P53 and MDM2
The tumor suppressor p53 plays a critical role in maintaining genomic stability and tumor prevention (50). The p53 pathway is tightly regulated by a number of proteins, including the critical negative regulators MDM2, MDM4, and WIP1 (50). Extra gene copies of MDM2 (22%), MDM4 (30%), and WIP1 (51%), have been reported in PanNET, which may lead to an attenuated p53 function (51). Thus, development of MDM2 inhibitors and related molecules can restore p53 tumor suppressor function and may provide tangible therapeutic options for PanNETs.

Cyclin dependent kinases and Rb
Cyclin-dependent protein kinase 4 (CDK4) and 6 are involved in phosphorylation of the Rb tumor suppressor gene leading to inactivation (52). Loss of Rb function results in unchecked transcriptional activation that can occur either by loss of Rb protein itself via RB1 gene mutations or by aberrations in other regulatory elements of the Rb pathway that increase phosphorylation of the Rb protein. In fact, 80% of cancers maintain an intact Rb protein but display genetic alterations of other components of the Rb pathway (53). CDK4/6 amplification and expression have been demonstrated in PanNETs as well as its activator, cyclin D (54). Loss of Rb
protein via RB1 gene mutation is also frequently observed in poorly differentiated and high grade NETs (55). Furthermore, growth of the human PanNET cell line QGP1 can be inhibited in a xenograft mouse model by the CDK4/6 specific inhibitor PD 0332991, which re-activates the Rb pathway (54). Thus, gene amplification and overexpression of CDK4 and CDK6 suggest that a subset of patients with PanNETs may respond favorably to CDK4/6 inhibitors that are currently entering clinical trials.

Biomarker discovery in neuroendocrine tumors

Circulating tumor cells

The CellSearch™ platform for cancer detection, amongst other technologies, has permitted research into circulating tumor cells (CTCs) as prognostic biomarkers in a number of malignancies (56). This platform requires the expression of epithelial cell adhesion molecule (EpCAM), a transmembrane glycoprotein, in order to isolate CTCs. EpCAM expression has been demonstrated in all midgut and PanNETs with variable expression in bronchial NETs (57). CTCs have been reported in blood samples from a number of patients with metastatic NETs, including pancreas, midgut and bronchial NETs, the latter having the highest levels (57). The same study reported no correlation between Ki-67 and CTC count nor a relationship between chromogranin A (CgA) and CTC count.

Since CTCs are shed into the circulation, studies are required to investigate the relationship between CTCs and angiogenesis. Discordance of Her-2 expression between CTCs and primary tumor in breast cancer (58) and differential expression of synaptophysin and CD56 in CTC in NETs (57) suggest CTCs may be heterogeneous. In the study discussed above, 82% of CTCs in NETs expressed synaptophysin and 21% expressed CD56, suggesting CTCs in NETs may be
heterogeneous (57). This heterogeneity may have implications as mutations may arise when shed from the primary tumor or could occur ‘de novo’ in the circulation, the latter possibly as an escape mechanism from therapy. For example, in lung cancer patients treated with anti-epidermal growth factor receptor (EGFR) therapy, a resistance-associated EGFR mutation emerged in CTCs (59). Thus, molecular characterization of CTCs could potentially assist in understanding NET metastasis and resistance to therapy in addition to their utility as biomarkers. Drugs chosen based on primary tumor markers may be ineffective against CTCs and the tumors they seed. Future implications include harvesting CTCs and testing chemotherapeutic agents in situ to assay drug susceptibility (60). Using CTCs to investigate the pathogenesis of NETs is advantageous with the capability of being frequently sampled throughout the disease course; they represent tumor tissue and obtaining CTCs with a blood test is less invasive than tumor biopsy.

It is only recently that cancer stem cells (CSCs) have been identified in NETs. In this study, aldehydride dehydrogenase+ (ALDH+) cells, when cultured from tumor tissue, were highly tumorigenic compared with ALDH– cells from the same tissue (44). It is unclear whether some CTCs identified by the CellSearch™ system are indeed CSCs. In animal models, aggressive CTCs have been shown to colonize their tumor of origin, in a process termed ‘tumor self-seeding’. This may explain relationships between tumor size, vascularity and prognosis and local recurrence seeded by disseminated cells following complete excision (61).

CTCs are associated with progressive NETs and could be used as a prognostic marker (57). In a prospective study of 120 patients with metastatic NETs, presence of CTCs conferred poorer overall survival with a hazard ratio of 14 (62). Furthermore, a reduction of CTCs at 3-5 weeks post-treatment predicted response to
therapy in addition to better survival compared with those in whom CTCs increased (63). Thus, patients on ineffective or potentially toxic therapies can have their treatment changed appropriately and their management tailored according to CTC changes.

**New biomarkers – an unmet need**

Sensitive and specific biomarkers are still largely lacking, and current assays fail to identify biomarkers at an early stage in disease progression. Studies that use proteomics and tissue arrays are needed to develop new biochemical and tissue specific markers. Global transcriptome analysis provides valuable information about the expression of genetic variants within cancer cells. An effective strategy may be to measure circulating mRNA to detect disease and perform amplification with real-time quantitative polymerase chain reaction (RT-PCR) to construct a gene terrain map that can be used to discriminate cell type and identify genes and mutations with prognostic potential (64). MiRNA-133a was downregulated during progression from primary to metastatic carcinoid tumor, suggesting that it may have an important role in carcinoid tumor development and progression with utility for diagnosis and/or prognosis (65).

**Novel methods of biomarker analysis**

Cancer companion diagnostics is a pathway-oriented approach to cancer analysis that integrates mutational analyses with protein activity biomarkers in clinical samples from patients treated with molecularly directed drugs. The goal is to develop diagnostics that better predict patient response to a drug and to create improved methods for monitoring therapy. Padlock probes, like selector probes, are highly
selective probes that generate circular molecules by target-dependent ligation upon perfect hybridization to pairs of sequences within a targeted molecule. The padlock probe technique enables detection of single nucleotide variants and other tumor or cell-specific transcriptomic markers in tissue specimens in situ at the single cell level (66).

The proximity ligation assay (PLA) is an immunoassay for protein analysis for the measurement and characterization of proteins with high specificity and sensitivity via DNA ligation and amplification reactions. The method relies on converting detection reactions to DNA reporter sequences. (Figure 3). The sensitivity of the PLA technique for plasma protein measurement renders the technique suitable for detection and identification of rare protein molecules in blood, potentially allowing for new classes of promising biomarkers to be identified from biobanked samples. The PLA method can also be combined with standard fluorescent microscopy for detection of proteins or protein–protein interactions and posttranslational protein modifications as activation markers in tissue sections using in situ PLA (67).

Future Directions
The efficacy of VEGF pathway and mTOR inhibitors in NET suggests that the molecular characterization of VEGF and mTOR pathway components in NETs may shed light on predictive markers, as well as mechanisms by which treatment resistance can be overcome. The discovery that some SST receptors are truncated, resulting in aberrant signaling, suggests that more detailed examination of SST receptor status in tumor tissue may also offer the opportunity to tailor more selective and effective treatments.
Remarkably, exome sequencing of PanNETs has suggested additional pathways of interest; in particular, these studies revealed a high prevalence of mutations in DAXX and ATRX genes implicated in chromatin remodeling. How these mutations correlate with the biologic or clinical characteristics of NET remains unknown and is the subject of ongoing studies, although these observations suggest that epigenetic changes may play a key role in NET growth. The development of high-throughput sequencing technology and other analytic techniques has facilitated the large-scale analysis of tumor tissue and other biospecimens and is likely to provide an effective way to correlate molecular features with clinical behavior.

Finally, the development of novel biomarkers in NET is likely to facilitate both patient care and the discovery of novel treatment targets. CTCs have been identified in NET patients, where they appear to have prognostic significance. Analysis of such cells may provide a practical way to assess biological changes that occur in tumors over time, and during treatment. Circulating MiRNAs in NET patients may also prove to be effective predictive and prognostic biomarkers to the extent that the detection of specific MiRNAs may reflect underlying tumor biology and may further point the way to novel pathways and targets.

Figure legends

Figure 1. Modes of resistance to anti-angiogenic therapy. On the one hand, intrinsic resistance or refractoriness defined as total lack of response to anti-angiogenic therapy. The specific mechanisms of such resistance include the multiplicity of pro-angiogenic factors expressed in tumors and vascular co-option. Thus, therapy is unable to reduce or stabilize tumors and there is no benefit from antiangiogenic
therapy. On the other hand, *acquired resistance* refers to the adaptive capacity presenting tumors leads to evade the therapeutic blockade after an adequate effectiveness phase. Induced adaptive mechanisms, including overexpression of alternative pro-angiogenic factors, recruitment of vascular progenitor cells (BMDCs) and pericyte coverage, ultimately allow for revascularization despite of therapy blockade, allowing tumor regrowth and disease progression.

**Figure 2.** Schematic representation of the mTOR pathway and associated regulatory circuitries. Mammalian target of rapamycin (mTOR) exists as two different complexes (mTORC1 and mTORC2) that are activated through different signaling cascades. Here is depicted the activation of mTORC1 by receptor tyrosine kinases triggered signaling. Positive and feedback regulatory loops are also described. PIP2, phosphatidylinositol (4,5)-biphosphate; PIP3, phosphatidylinositol (3,4,5)-triphosphate.

**Figure 3.** Scheme of proximity ligation in situ assay. A) Primary probes (primary antibodies) bind to target proteins. B) Secondary probes (secondary antibodies) with conjugated oligonucleotides recognize the primary probes. C) Additional oligonucleotides are hybridized and ligated to form circular DNA molecule. D) The newly formed DNA molecule can be amplified by rolling circle amplification. A concatemeric amplification product can be detectable by hybridization of fluorescently labeled oligonucleotides.

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Kjell Oberg has taken part in advisory board meetings and acted as a speaker for Novartis, Ipsen and Pfizer

Oriol Casanovas has received consultancy fees from Teva and Ipsen and speaker fees from Ipsen and Pfizer

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Daniel Chung has acted as a consultant for Ipsen

Gianfranco Dellefave has taken part in advisory board meetings for Ipsen

Mohid Khan has received conference and speakers fees from Novartis and Ipsen

Matthew Kulke has acted as a consultant for Ipsen, Novartis, Lexicon and Pfizer

Aldo Scarpa has taken part in advisory board meetings for Ipsen

Laura H. Tang has taken part in advisory board meetings for Ipsen

Bertram Wiedenmann has received conference and speakers fees from Novartis and Ipsen
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Table 1. Driver gene mutations and cancer

<table>
<thead>
<tr>
<th>Cancer</th>
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Abbreviations: GIST, gastrointestinal stromal tumor; CML, chronic myeloid leukemia
Figure 1:

Intrinsic resistance

Acquired resistance

Anti-VEGF/R therapy

Vascular cooption

High expression of VEGFs, FGFs, angiopoietins, ephrins, etc.

Response phase

Unresponsive to therapy

Resistance phase

BMDCs recruitment

Pericyte coverage

Upregulation of VEGFs, FGFs, angiopoietins, ephrins, etc.
Figure 2:
Figure 3:
Molecular Pathogenesis of Neuroendocrine Tumors: Implications for Current and Future Therapeutic Approaches

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