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TOPIC

Molecular pathogenesis and targeted therapy of sporadic pancreatic neuroendocrine tumors

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Abstract Over the past few years, knowledge regarding the molecular pathology of sporadic pancreatic neuroendocrine tumors (PNETs) has increased substantially, and a number of targeted agents have been tested in clinical trials in this tumor type. For some of these agents there is a strong biological rationale. Among them, the mammalian target of rapamycin inhibitor Everolimus and the antiangiogenic agent Sunitinib have both been approved for the treatment of PNETs. However, there is lack of knowledge regarding biomarkers able to predict their efficacy, and mechanisms of resistance. Other angiogenesis inhibitors, such as Pazopanib, inhibitors of Src, Hedgehog or of PI3K might all be useful in association or sequence with approved agents. On the other hand, the clinical significance, and potential for treatment of the most common mutations occurring in sporadic PNETs, in the MEN-1 gene and in ATRX and DAXX, remains uncertain. The present paper reviews the main molecular changes occurring in PNETs and how they might be linked with treatment options.

Keywords Genetics · Molecular · Mutation · Pancreatic neuroendocrine tumors · Targeted therapy

Introduction

In the past decade, knowledge regarding molecular pathology of sporadic pancreatic neuroendocrine tumors (PNETs) has increased substantially, thanks to microarray studies and novel mutational analysis methods [1]. Over the same period of time, a number of targeted agents have been tested in clinical trials in this tumor type. For some of these agents there is a strong biological rationale. Among them, the mammalian target of rapamycin (mTOR) inhibitor Everolimus (Afinitor, Novartis, Basel, Switzerland) and the antiangiogenic agent Sunitinib are approved for the treatment of PNETs. However, there is lack of knowledge regarding biomarkers able to predict their efficacy, and about mechanisms of resistance to these targeted agents [2]. It has also been ascertained that the most common mutations in sporadic PNETs are of the multiple endocrine neoplasia type 1 (MEN1) gene and of the genes ATRX and DAXX. However the clinical significance and potential for treatment of these mutations are uncertain. The present paper will review the main molecular changes occurring in PNETs and how they might be linked with treatment options.

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MENIN

MENIN is a nuclear protein, encoded by the MEN1 gene, which regulates gene transcription by coordinating chromatin remodeling. It is involved in the negative modulation of cell cycle inhibitors, such as p27KIPI and p18INK4c, of transcription factors such as SMAD3 and JUND, and interacts with the DNA repair machinery [3]. MENIN is considered a tumor suppressor, although its exact role is not completely clear and its action is often controversial. In fact, MENIN works as an inhibitor of proliferation, maintaining the promoter activity of CDKN2C (p18) and CDKN1B (p27) through H3K4 methylation, thus regulating the expression of cell cycle progression inhibitors [3, 4]. Nevertheless, under physiological or pathological conditions, such as obesity or pregnancy, MENIN stimulates pancreatic endocrine cell proliferation controlling G1 to S progression [5, 6].

While the role of MENIN has been extensively investigated in patients with MEN1 syndrome, mutations of the MEN1 gene have also been found in about 25–44% of sporadic PNETs [7, 8], suggesting a role in the pathogenesis of these tumors. Interestingly, MEN1 mutations have been associated with prolonged survival in patients with metastatic

disease [8]. Although mutations of MEN1 are the most frequent alterations in sporadic PNETs, this does not represent a target for treatment yet, although there have successful attempts of gene therapy to replace the MEN1 gene in PNETs models. However, the relation between MENIN and other "druggable" pathways is gaining increasing interest. MENIN is able to suppress Akt activity reducing its translocation from the cytoplasm to the cell membrane during stimulation with growth factors, therefore reducing Akt-induced proliferation and anti-apoptosis activity [9]. On the other hand, MENIN expression is modulated by PI3K/Akt activity, which leads to phosphorylation of the transcription factor Foxo1, which in turn negatively regulates the expression of MENIN, enhancing proliferation [5, 10].

MENIN has also a role in preventing the RAS-driven activation of the mitogen-activated protein kinase (MAPK) pathway, leaving the RASSFA inhibitory pathway intact. The removal of this mechanism of MAPK pathway blockage could explain why the loss of MENIN causes proliferation in PNETs [11]. However, targeting the RAF or MAPK pathway alone does not seem a promising strategy in PNETs [12]. Recently, a novel link between MENIN and the Hedgehog (Hh) signaling pathway has been suggested by Gurung et al. [13]. Furthermore, Fendrich et al. showed how Cyclopamine, a Hedgehog inhibitor, decreased tumor cell proliferation, reduced tumor volume and significantly prolonged median survival in the transgenic mouse model of PNET [14]. The orally available smoothened antagonist LDE225 is therefore under investigation for PNETs. Finally, a valuable target therapy for MENIN could possibly arrive with RNA antagomir(s)-based strategies, as suggested by Luzi et al., but further studies are needed to explore this field [15].

ATRX and DAXX and epigenetics changes in PNETs

In the past few years, the possibility to use novel technologies of DNA sequencing has led to a dramatic progress of knowledge of the mutational status of human cancers. Next-generation sequencing permits to identify single nucleotide mutations of either the whole genome or of the exomes. The first application of high throughput exomic sequencing of PNETs [8] demonstrated that, after those of the MEN1 gene, the most common mutations were of the alpha-thalasemia/mental retardation syndrome, X-linked (ATRX), and death domain-associated protein (DAXX) genes. Of 68 PNETs cases investigated, 42% had mutation of one of these two genes, and these were mutually exclusive (Table 1). The mutations were associated with better clinical outcome. These findings have raised interest on the significance of ATRX and DAXX in PNETs. ATRX is a nuclear protein belonging to the family of chromatin remodeling proteins, whose germinal mutations cause the alpha-thalasemia syndrome [16]. ATRX is able to recognize the methylation status of histones, and cooperates with DAXX in determining histones deposition.

Subsequent studies have confirmed that ATRX and DAXX mutations are frequent in PNETs, and that mutations correspond with decreased protein expression at immunohistochemistry [17–21].

The mechanism by which the loss of ATRX or DAXX favors tumor cells seems related with telomerase activity. Over the course of life, telomeres progressively become shorter with cell division, and the progressive loss of telomeric DNA leads to cell senescence and death. Tumor cells develop mechanisms of resistance to senescence, including the reactivation of telomerase [22]. However, some tumors lack telomerase activity, and display another, telomerase-independent, peculiar mechanism of resistance to cell senescence and death, named "Alternative Lengthening of Telomeres" (ALT) [23]. Interestingly, human cancer cell lines that display the "ALT pathway" are characterized by ATRX mutations [24]. The association between ATRX/DAXX mutations or their decreased expression, and the "ALT pathway" was confirmed by three different studies conducted in sporadic or MEN1 associated PNETs [17, 18, 21]. In the last of these studies, Marinoni et al. also associated ATRX/DAXX with chromosomal instability. A less recent study demonstrated the absence of telomerase activity in 27 PNET patients [25]. It is therefore likely, that PNETs cells tend to use almost exclusively the "ALT pathway" and not the telomerases to gain "immortality". Future studies evaluating whether PNETs without ATRX/DAXX mutations show telomerase activity or other molecular changes, such as mutations at TERT promoter might prove interesting.

Another puzzling finding is the lack of consistency regarding the association between ATRX/DAXX mutations and the clinical outcome of PNET patients (Table 1). The absence of a clear clinical significance and the difficulties in targeting the "ALT pathway" also limit the clinical potential of these findings. Indeed, while a number of agents, such as imetelstat, are able to target telomerase, there are few data regarding the development of efficient ALT inhibitors [26]. On the other hand, knowledge regarding ATRX and DAXX and their interaction with histones and telomeres has attracted many researchers, and more attention to modifications occurring in histones, chromatin remodeling, and epigenetics might help define other potential targets for treatment, as methylation of different genes has been associated with negative outcome in PNETs [27].

Angiogenesis in PNETs

A rich vascularization is a typical feature of PNETs, and often allows radiological differential diagnosis with pancreatic

Table 1 Summary of the main findings regarding mutations of DAXX and ATRX

Study (reference)	Method	PNETs	DAXX mutation/ expression	ATRX mutation/ expression	Relation between DAXX and ATRX	Association with other molecular alteration	Association with clinical outcome
Jiao [8]	Exomic sequencing and sequencing in validation set	68	25% mutated	17.6% mutated	Mutually exclusive mutations	–	Better outcome
De Wilde [17]	IHC	50 from 28 MEN1 patients	6% absent or defective IHC expression	2% absent or defective IHC expression	Concomitant in one case	ALT phenotype	Larger diameter and higher grade
Heaphy [18]	Sequencing	39	24%	22%	Mutually exclusive	ALT phenotype	–
Chen [19]	IHC	70	25% loss IHC expression	15.7% loss IHC expression	Mutually exclusive	–	No association with stage or grade
Yuan [20]	Sequencing	37	54% mutated	11% mutated	–	–	Worse outcome
Marinoni [21]	IHC (sequencing in a subgroup)	92]	25% loss IHC expression	18% loss IHC expression	4% concomitant loss	ALT phenotype and CIN	Worse outcome

ALT alternative telomerase length, *IHC* immunohistochemistry

adenocarcinoma. Interestingly, the importance of angiogenesis switch in human cancer has been extensively described in a PNET transgenic mouse model (Rip1-Tag2) [28]. The vascular endothelial growth factor (VEGF) and its receptor VEGFR, as well as the platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) are expressed in PNET cells and/or in the surrounding endothelia [29]. However, there is no clear correlation between the expression of VEGF or the microvascular density and the prognosis of PNET patients. Indeed, some studies have found that expression of VEGF correlates with a more aggressive tumor behavior [30], while others found that malignant tumors show lower VEGF expression than benign ones [31, 32]. These findings have been recapitulated under the term "neuroendocrine paradox" [33], and might be explained by the fact that intense microvascular density and VEGF expression are markers of "well-differentiated" neoplasms, resembling normal islet cells architecture. As pancreatic endocrine cells turn more aggressive and lose their original features, or gain further molecular changes, their rapid growth causes hypoxia, and activates the hypoxia-inducible factors-1α (HIF-1α) pathway, leading to an increase of endothelial proliferation eventually resulting in changes of the normal vascular architecture.

From a clinical viewpoint, however, inhibition of neoangiogenesis is a valid treatment approach for PNETs. The main employed agents are either tyrosine kinase inhibitors targeting the VEGFR and/or other receptors (PDGFR, FGFR) or antibodies directed against VEGF itself. Among tyrosine kinase inhibitors, the only one approved for clinical use in patients with advanced PNETs is Sunitinib (Sutent, Pfizer, New York, NY, USA), which has activity against multiple targets including VEGFR, PDGFR, c-KIT, Flt-3 and RET [34]. The efficacy and safety of Sunitinib had first been demonstrated in a phase II study including both PNETs and carcinoid tumors [35], with a median time to progression of 7.7 months for patients with PNETs. The drug has been approved after publication of a large randomized controlled trial of Sunitinib versus placebo. The study demonstrated an increased progression-free survival (PFS) in patients receiving Sunitinib (10.2 vs 5.4 months). Overall survival was also improved with Sunitinib. Severe side-effects (grade 3–4) were observed in 12% and 10% of patients, respectively [36].

Among other tyrosine kinase inhibitors, Sorafenib (Nexavar, Bayer Pharma AG, Berlin-Wedding, Germany), a multiple kinase inhibitor targeting VEGFR2, PDGFR, FGFR1 and RAF, has been poorly investigated as a single agent in PNETs. Its combination with both everolimus or bevacizumab in phase I and II trials showed clinical benefit but unfavorable safety results [37, 38].

Pazopanib (Votrient, Glaxo Group, London, UK) is an orally available angiogenesis inhibitor that targets VEGFR1, −2 and −3; PDGFRα and c-kit. Its activity as monotherapy has been demonstrated in a phase II study in metastatic gastroenteropancreatic neuroendocrine tumors. Stable disease was observed in 56.8% of cases and partial response in 18.9%, with 11% grade 3–4 toxicity [39]. The median PFS was 9.5 months. Interestingly, the activity of Pazopanib was significant also in patients pre-treated with targeted therapies.

Bevacizumab (Avastin, Roche, Basel, Switzerland) is a monoclonal antibody binding circulating VEGF, approved for the treatment of colorectal cancer. There are few data regarding Bevacizumab as monotherapy for PNETs. Some interesting results have been published for Bevacizumab-based combined treatments. The combination of Bevacizumab with temozolomide [40] resulted safe in a phase II study in progressive, pre-treated patients, with 86% response rate (partial response + stable disease) in PNETs and a PFS of 14.3 months. The combination of Bevacizumab with metronomic capecitabine and Octreotide also achieved interesting results in a phase II study of 45 patients, with partial response in 18% and a PFS of 15 months. PNETs seemed to respond better than non pancreatic tumors [41]. Bevacizumab seems an interesting agent for combination studies with other cytotoxic drugs.

There are no data regarding biomarkers predictive of response to antiangiogenic agents. There are also few molecular data explaining the occurrence of secondary resistance to antiangiogenic agents, but it has been reported that after initial response, evasive resistance might lead to a more aggressive behavior [42]. This phenomenon might rely on progressive selection of clones able to survive in a hypoxic environment, and might be limited by combined treatments targeting different players of the angiogenesis machinery.

The PI3K/Akt/mTOR pathway

The mammalian target of rapamycin (mTOR) is an intracellular serine/threonine kinase working as a transduction factor to which a wide variety of physiological and pathologic extra and intracellular signals converge. mTOR is involved in the regulation of cell metabolism, survival, proliferation and motility, through the regulation of protein translation. Its function is carried out through the formation of two complexes, mTORC1 and 2 [43]. mTORC1 regulates mRNA translation and protein synthesis in response to nutrients, hormones and growth factors. It acts through phosphorylation of the S6 kinase 1 (S6K1), which subsequently phosphorylates the S6 ribosomal protein (S6rp) of the 40S subunit, initiating protein synthesis, and the eukaryotic initiation factor 4E (eIF4E) binding protein (4EBP1), which, under normal conditions, inhibits cap-dependent translation by binding eIF4E. Once phosphorylated, 4EBP1 releases eIF4E and subsequently the cap-dependent mRNA translation of proteins initiates. This complex is sensitive to rapamycin and its analogs [44]. mTORC2 is involved in cell survival but also in cytoskeletal remodeling and cell migration. The main substrates of mTORC2 are protein kinase B (Akt) and protein kinase C. This complex is relatively insensitive to rapamycin [44–46]. The expression and activity of mTOR have been proved to be higher in PNETs tissue than in normal pancreatic islet cells. Expression of mTOR or of its activated downstream target p4EBP1 has also been associated with a higher proliferative index and shorter survival in patients with neuroendocrine tumors. The expression of mTOR, p-mTOR and S6K is significantly related to tumor aggressiveness in terms of mitotic count, tumor size, staging, vascular invasion and metastasis [47]. Furthermore, low levels of 4EBP1 or high levels of eIF4E are thought to confer resistance to rapamycin analogs [48]. Therefore, mTOR and its effectors might be biomarkers of aggressive disease (Table 2), but are not mutated in PNETs, with most molecular changes occurring upstream on the PI3K/Akt/mTOR pathway.

Table 2 Summary of available data regarding expression, role and potential as biomarker of genes of the mammalian target of rapamycin (mTOR) pathway

Gene	Role in the mTOR pathway	Percentage of mutations in sporadic PNETs	Expression in PNETs	Related outcome	Therapeutic biomarker	Therapeutic target
PTEN [8, 17, 29, 49–51]	Negative regulator	10–29%	Reduced	Worse OS and PFS if low	–	–
TSC2 [8, 17, 29, 51]	Negative regulator	8.8%	Reduced	Worse OS and PFS if low	–	–
p-mTOR [29, 47]	Read-out	0%	Increased	Higher Ki-67, worse OS, worse staging	Yes	Yes, approved
PI3K [8, 17, 29]	Positive regulator	1.4%	–	–	–	Yes, under investigation
p-S6K [29, 47]	Read-out	0%	Increased	Higher Ki-67, worse OS, worse staging	Possibly	Possibly
p-4EBP1 [48]	Read-out	–	Increased	Worse OS, resistance to rapamycin analogs if low	–	–
p-Akt [29, 54, 55]	Positive regulator	0%	Increased	–	Possibly	Possibly, under investigation

OS overall survival, *PFS* progression-free survival

PI3K is a complex encoded by the PIK3CA gene and works downstream of many growth factors, such as insulin or insulin-like growth factors (IGF-1) and their receptors. Upon phosphorylation of receptor's substrates, such as IRS, PI3K is recruited to the cell membrane and turns phosphatidylinositiol-4,5-bisphospate (PIP2) to phosphatidlinosiytiol-3,4,5-bisphospate (PIP3), and eventually these changes result in activation of Akt. In this process, the antagonist of PI3K is PTEN, a phosphatase that turns PIP3 to PIP2. PTEN is mutated or lost in about 10–29% of sporadic PNETs [49, 50]. The expression of PTEN in PNETs has been positively correlated with longer survival especially when correlated with low expression of p-mTOR [49]. The PI3K-Akt pathway is linked to mTOR through the tuberous sclerosis proteins TSC1 (hamartin) and TSC2 (tuberin) that act as negative regulators of the mTOR signaling: when phosphorylated by Akt TSC2 gets inactivated, unblocking the mTOR activator GTPase Rheb.

In sporadic PNETs, somatic mutations of genes involved in the mTOR pathway has been identified in 16% of cases, including PTEN, TSC2 and PIK3CA [17, 29]. Particularly, PTEN was found mutated in 7%, TSC2 in 8.8% and PIK3CA in only 1.4% [8]. Moreover, lower protein levels of TSC2 and PTEN are related to shorter disease-free and overall survival [51]. All these findings support the relevance of the mTOR pathway as potential therapeutic target for PNET (Table 2). However, biomarkers to select individual patients who would respond to treatment with mTOR inhibitors are lacking.

The activity of the mTORC1 inhibitor Everolimus on PNETs has been explored in the RADIANT-1 phase II trial, on 160 patients, 45 of whom also received concurrent treatment with octreotide. The combination of the two drugs increased the median PFS compared to those receiving everolimus alone. In the RADIANT-3 trial, a phase III placebo-controlled trial of 410 patients with advanced PNETs, Everolimus monotherapy was compared with placebo. The results showed a significant prolongation in median PFS in the Everolimus arm (11 vs 4.6 months). The result of this trial led to approval of Everolimus by FDA and EMA for the treatment of locally advanced, metastatic or unresectable PNETs [52]. The most common grade 3/4 adverse events were stomatitis (7%), anemia (6%), hyperglycemia (5%), and pneumonitis. Similar results in terms of activity and adverse events have been reported in non-controlled studies [53].

Temsirolimus is another rapamycin analog, also evaluated in a phase II study on 37 patients with advanced, progressive neuroendocrine tumors, with good results. About 50% of patients achieved stable disease with a median PFS of 6 months. It also appeared more active in PNET patients compared to carcinoid (median PFS 10.6 months in PNETs).

Primary or secondary resistance to treatment with Everolimus has been linked with escape routes activating the upstream PI3K/Akt pathway. These are thought to be due to inhibition of mTORC1 with consequent upregulation of PDGFRs and IRS-1 through a negative feedback loop leading to Akt activation. Rapamycin and Everolimus use, indeed, has been found to increase Akt phosphorylation [54]. Although phosphorylation of Akt is considered a part of the evasive response to mTOR inhibitors, it has also been reported that upon treatment with Everolimus, patients experiencing a partial response are more likely to have an increase in p-Akt, both before and during treatment. Further studies should confirm if p-Akt might serve as a biomarker of sensitivity to Everolimus rather than of secondary resistance [55].

Different strategies might overcome secondary resistance to Everolimus. One is the combination of Everolimus with other drugs, either acting on the same pathway (vertical inhibition) or targeting another pathway (horizontal inhibition). Another option could be a sequential treatment, after failure of the first one. A logical strategy to overcome resistance to mTOR inhibitors is, indeed, to use other inhibitors able to block targets upstream of mTOR, such as Akt or PI3K. The PI3K-inhibitor that attracted researchers the most was BEZ235 for its dual inhibition activity of PI3K and mTORC1/2. In a recent study, a combined treatment with BEZ235 and Everolimus at low doses resulted as more effective compared to the use of each drug alone, and was active in cell line models of both primary and secondary resistance to Everolimus [56]. However, clinical trials testing BEZ235 efficacy and safety compared to Everolimus, or its activity after failure of Everolimus, have been stopped for side-effects.

Other "druggable" molecular alterations

Another regulator of the PI3K/Akt and the MAPKs pathways is EGFR (ErbB-1), a member of the ErbB family of tyrosine kinase receptors. PNET patients with activated EGFR exhibit a worse prognosis [57]. EGFR activation results in a concomitant upregulation of downstream effectors such as Akt and ERK [58]. More recently EGFR transactivation induced by various GI hormones/neurotransmitters and mediated by Src, was proven to have a role in PNET cells growth [59]. Gefitinib is a small molecule inhibitor of EGFR intracellular domain. *In vitro* studies showed its activity with a dose-dependent reduction of cell viability and increased apoptosis in PNET cells [60]. Clinical studies with gefitinib showed a 30–60% of PFS at 6 months, though with rare remissions [61]. Other drugs directed towards this target are the anti-HER-2 antibody Pertuzumab or the anti-EGFR Erlotinib, but phase II trials with pertuzumab and erlotinib were terminated due to extreme toxicity.

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. SFKs were found

to be overexpressed in PNETs [62] and have a role in EGFR transactivation [59]. Inhibiting its activity decreases adherence, spreading and migration of PNET cells [63]. Furthermore, SFKs control mTOR activity during adhesion and concurrent inhibition of SFKs and mTOR reduced proliferation of PNET cells without inducing PI3K/Akt activity [64]. It was also found how Src has a role in stem cells isolated from intestinal carcinoid cells [65], confirming its likely relevant role as possible co-treatment.

Conclusion

As compared with only a decade ago, the knowledge regarding the molecular pathogenesis of sporadic PNETs has substantially improved, in parallel with an increase of high quality clinical studies exploring "targeted" agents. However, there is a lot to do, to obtain reliable *in vitro* models on which accurate preclinical investigations might be performed. Such studies should help define whether the combination of different drugs, or a rational sequence of different targeted agents may overcome acquired resistance to single targeted agents. Finally, biomarkers predictive the sensitivity or resistance of individual patients in clinical practice are lacking, and knowledge on the role of the most common mutations is unclear.

Conflict of interest None declared.

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