Neuroendocrine Tumors of the Pancreas: Molecular Pathogenesis and Perspectives on Targeted Therapies

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Abstract: Pancreatic neuroendocrine tumors (PNETs) are a heterogeneous group of neoplasms that are the second most common among pancreatic neoplasms. Treatment of PNETs appears to be quite difficult because diagnosis in many patients occurs only at the latest stage when distant metastases are recognized. Therefore, treatment with drugs targeting PNET oncogenesis is a promising strategy in such patients. In this work, we review the present knowledge on the molecular nature of PNETs, and the genetic basis of PNET-associated hereditary syndromes, including multiple endocrine neoplasia type I, von Hippel–Lindau disease, neurofibromatosis type I, and tuberous sclerosis. In addition, the results of phase III, randomized, placebo-controlled trials of the efficacy of everolimus and sunitinib for treatment of extensive non-resectable PNETs are reviewed.

Keywords: Neuroendocrine tumor, multiple endocrine neoplasia type I, von Hippel–Lindau disease, neurofibromatosis type I, tuberous sclerosis, targeted therapy, everolimus, sunitinib.

INTRODUCTION

Pancreatic neuroendocrine tumors (PNETs) are a heterogeneous group of neoplasms that are the second most common among pancreatic neoplasms [1]. The annual incidence, which has maintained a steady upward trend, is approximately 1–4 cases per million [2, 3]. To achieve the best clinical outcomes in patients with PNETs, a multidisciplinary approach with the assistance of a variety of clinicians, including oncologists, gastroenterologists, endocrinologists, radiation therapists, and surgeons, is required. However, treatment of PNETs appears to be quite difficult because diagnosis in many patients occurs only at the latest stage when distant metastases are recognized [1, 4]. In such cases, the efficacies of systemic chemotherapy and radiological and surgical technique combinations are severely reduced. Therefore, treatment with drugs targeting PNET oncogenesis is a promising strategy in such patients [4, 5].

MOLECULAR MECHANISMS OF PNET DEVELOPMENT

PNETs are based on genetic mutations that cause uncontrolled proliferation of hormone-responsive cells [6, 7]. Numerous studies have shown that mutations of common oncogenes (Ras, c-Myc, Jun) and common tumor suppression genes (p53, Rb) are less typical and occur rarely in PNETs than in the most frequent human tumors [2, 7-10]. At present, the oncogenic mutations in sporadic PNETs have not been studied in depth. Recent research has suggested that PNETs are characterized by mutations in tumor suppressor genes (*INK4a*, *Smad4*, *MEN1*), mutations in chromatin remodeling pathway genes (*ATRX*/*DAXX*), amplification of the proto-oncogene HER2/neu (ErbB-2), and increased expression of some growth factors and/or growth factor receptors [2, 7, 10-13].

The *INK4a* gene alterations are present in 52%–90% of all PNETs cases [2]. Normally, the *INK4a* gene encodes the p16INK4a tumor suppressor protein that binds the cyclin-dependent kinases 4 (CDK4) and 6 (CDK6), which prevents their functionally active complexation with cyclin D. Inactivation of *INK4a* mutations affects p16INK4a protein function. Moreover, the functionally active complex (cyclin D-CDK4/6) phosphorylates the retinoblastoma protein, which weakens its repressor connection with the E2F transcriptional factor. Unconjugated E2F initiates cell cycle passage into the S-phase through interaction with other transcriptional factors (Figure **1**) [12, 14-17].

Another mechanism of oncogenesis involves mutations in the *Smad4* tumor suppressor gene that encodes the homonymous SMAD family protein, which is involved in transforming growth factor β (TGF-β)-inducible signaling pathways. Normally, TGF-β (depending on the target cell) operates by inhibiting cell proliferation, cell cycle arrest in the G1 phase, and apoptosis induction. Attachment of TGF-β to its membrane receptor type 2 (TGF-β-R2) induces receptor type 1 (TGF-β-R1) recruitment and heterotetramer complex formation. Further signaling is initiated by phosphorylation of TGF-β-R1 followed by

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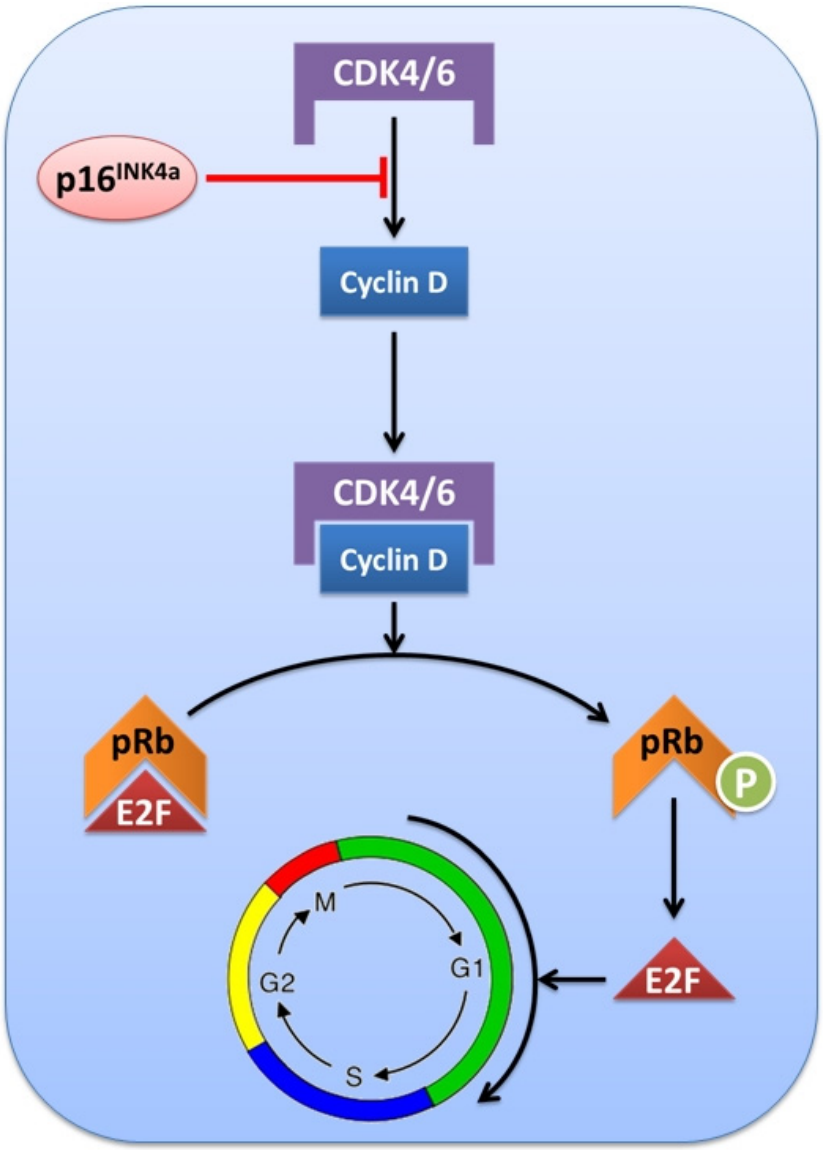

Figure 1: Role of p16INK4a protein in cell cycle regulation.

p16INK4a binds to the cyclin-dependent kinases 4 (CDK4) and 6 (CDK6), which prevents the formation of its functionally active complexes with cyclin D.

Note: CDK4–cyclin-dependent kinase 4, CDK6–cyclin-dependent kinase 6, pRb–retinoblastoma protein, P–phosphorylation, E2F–transcription factor E2F.

phosphorylation of SMAD2 or SMAD3 (R-SMAD) receptor-regulated proteins. Phosphorylated R-SMAD forms a complex with the SMAD4 protein that enters the nucleus, where it functions as a transcription factor and regulates target gene expression. The subsequent activation of the p15Ink4b, p27Kip1, and p21Waf1/Cip1 CDK inhibitory genes results in cell cycle arrest in the G1 phase (Figure **2**). Inactivating mutations in the Smad4 gene cause abnormalities in TGF-β receptor signal transduction and block its tumor suppressive potential [16, 18-20].

Somatic mutations in the genes *ATRX* (α thalassemia / mental retardation syndrome X-linked) and *DAXX* (death-domain-associated protein), encoding two subunits of a chromatin remodeling complex, are identified in 43% of the PNETs [11]. ATRX and DAXX proteins are required for the incorporation of H3.3 at telomeres. Mutations in these genes are strongly associated with alternative lengthening of telomeres. Such mutations are associated with longer survival and better prognosis [11].

Amplification of the *HER2/neu* proto-oncogene possibly leads to initiation of some signaling pathways that culminate in proliferation and cell cycle dynamics and have been detected in various tumors. In

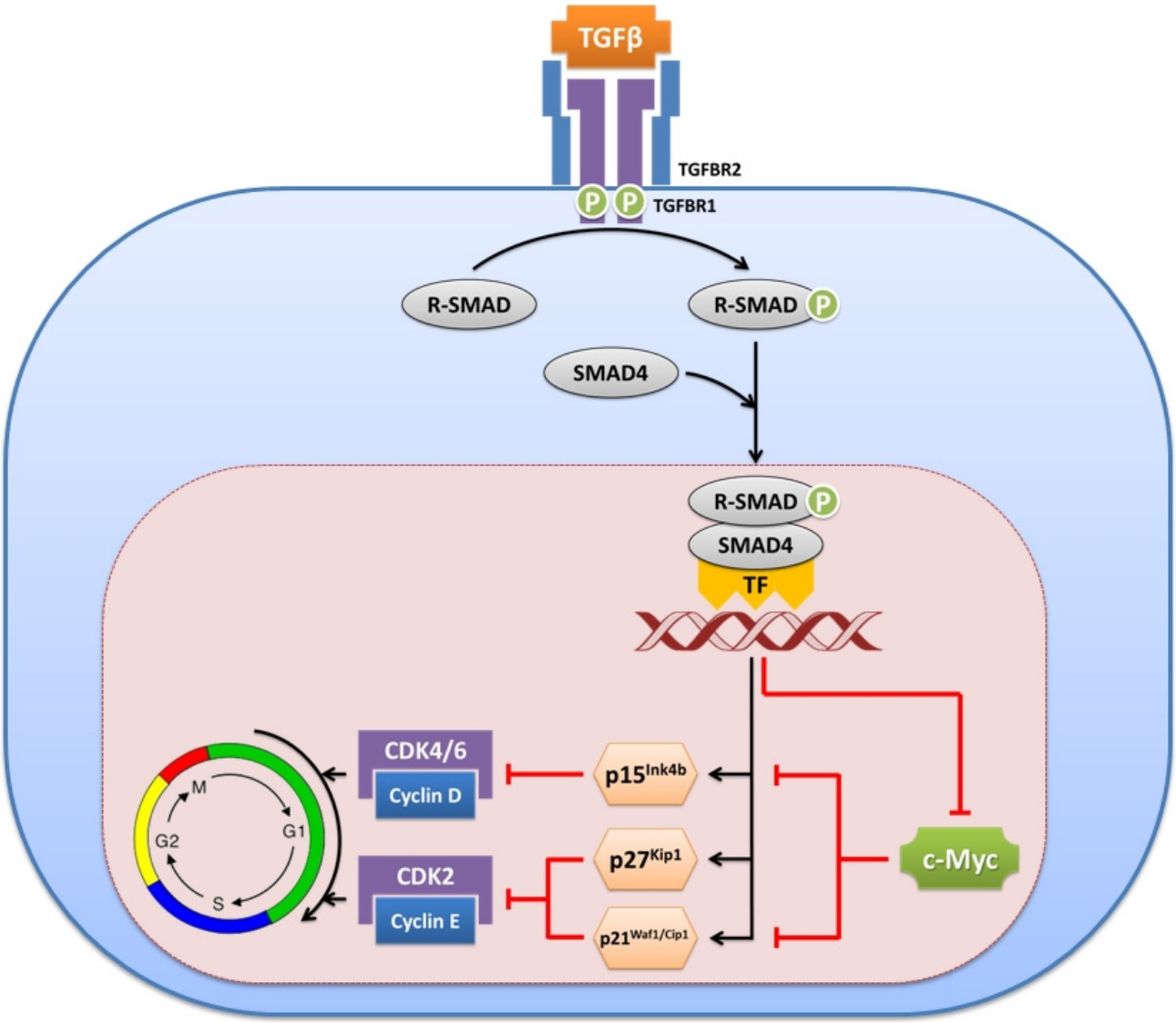

Figure 2: Role of SMAD4 protein in cell cycle regulation.

TGF-β signaling is initiated by phosphorylation of TGF-β-R1, followed by phosphorylation of SMAD2 or SMAD3 (R-SMAD) receptor-regulated proteins. Phosphorylated R-SMAD and SMAD4 form a complex that enters the nucleus, where it functions as a transcription factor and regulates target gene expression.

Note: TGFβ–transforming growth factor β, TGFBR1–transforming growth factor β receptor type 1, TGFBR2–transforming growth factor β receptor type 2, R-SMAD–receptor-regulated SMAD proteins, P–phosphorylation, TF–transcription factor, CDK2–cyclin-dependent kinase 2, CDK4–cyclin-dependent kinase 4, CDK6–cyclin-dependent kinase 6.

particular, such signaling pathways include those involving phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) and Ras/Raf/MEK/MAPK [7, 16, 21-23]. Therefore, for gastrinomas, amplification of *HER2/neu* has been found in 14%–45% of all cases and often is associated with multifocal or metastatic patterns of the disease [24, 25].

Overexpression of the growth factors and their receptors in PNETs has been actively studied over recent decades. The surface of PNET cells consists of some types of growth factor receptors, including those with tyrosine kinase activity, such as insulin-like growth factor 1 receptor (IGF-1R), epidermal growth factor receptor (EGF-R), and stem cell factor (SCF) receptor c-KIT [7, 12, 13, 16, 26, 27]. IGF-1R hyperexpression level has been shown to correlate with tumor aggression and metastatic activity in a study by Furukawa *et al*. [28]. In addition, Peghini *et al*. [29] reported that in gastrinomas EGF-R was overexpressed in 18% of all cases and often correlated with the presence of metastatic disease and with negative clinical prediction. As a type III receptor with tyrosine kinase activity, c-KIT binds the SCF ligand. Pathological expression of this receptor has been

defined in many tumors and has predictive importance [7]. Recently, the prognostic significance of c-KIT expression has been evaluated in patients with various PNETs because early studies reported contradictory results [26, 27, 30, 31]. In particular, significant correlation of c-KIT overexpression with tumor aggression and low survival rates was shown in a study by Zhang *et al*. [32].

In 14% of sporadic PNET cases, mutations were identified in the genes that regulate the PI3K/AKT/mTOR signaling pathway, including *TSC2*, phosphatase and tensin homologue (*PTEN*), and PI3K catalytic subunit alpha (*PIK3CA*) [11].

Thus, many heterogeneous cytogenetic defects contribute to the development of sporadic PNETs. At present, the mechanism of the interaction of different mutations involved in cellular neoplastic transformation remains unknown. It appears that mutations in the regulators of cyclin-dependent kinases, the PI3K/AKT/mTOR signaling pathway, and amplification of multiple receptor tyrosine kinases lead to increased cell proliferation, cell survival, and angiogenesis. Growth factors may initiate an autocrine activation of the PI3K/AKT/mTOR signaling pathway. In turn, *MEN1* gene mutations cause many cytogenetic defects, including the suppression of DNA repair. Further research is needed in this direction.

PNET-ASSOCIATED HEREDITARY SYNDROMES

Defined PNETs develop in frames of hereditary syndromes with a recently clear genetic basis (Table **1**) [33].

Nearly 10% of PNET cases develop under multiple endocrine neoplasia syndrome type 1 (MEN-1). MEN-1 syndrome (Wermer's syndrome) is a group of disorders with an autosomal dominant mode of inheritance that occurs when tumors are found in at least two endocrine glands with contemporary embryonic connections to neuroectoderm [6, 34]. This syndrome is associated with inactivating mutation of the *MEN1* gene and was mapped in the pericentric region of chromosome 11 (11q13 loci) in 1988 [35]. To date, literature data on more than 1336 *MEN1* gene mutations have been identified [36]. Such phenotypic variability is randomly determined by damage in the second allele of the *MEN1* gene, as stated in Knudson's two-hit model of oncogenesis [37]. In germinal cell lines, the *MEN1* gene mutation inherited from parents (inherited form) or acquired in the early embryonic period (sporadic form) is a first hit under this hypothesis. The second hit is a somatic mutation in the second *MEN1* gene allele. This mutation, in particular, causes high variability in organs and their systems and is involved in pathological processes and in onset age [33, 34]. It is interesting to note that somatic mutations of the *MEN1* gene have been found in nearly 33% of patients with sporadic PNET forms [38]. The product of the *MEN1* gene is a 610-amino acid tumor suppressing protein, menin, which regulates different cell and genomic homeostasis functions. In particular, menin modulates the activity of cell cycle inhibitors and at the nuclear level, inactivates transcription factors and contributes to DNA reparation processes [39-42].

In cooperation with the mixed lineage leukemia protein complex, menin regulates the expression of inhibitors of the cyclin dependent kinases, p18INK4C and p27Kip1, which are responsible for the resistance to formation of the functionally active cyclin D-CDK4/6 and cyclin E-CDK2 complexes, respectively [42-44]. Menin and kinase Cdc7 complex formation is prevented by interaction of the C-terminal domain of menin with the DBF4 (ASK) protein. This complex (kinase Cdc7–DBF4) is essential for initiation of DNA replication during the S-phase of the cell cycle [42, 45, 46]. Inhibition of nuclear factor κB and transcriptional factor JunD (in the latter case with the mSin-histone deacetylases complex) transcriptional activity mediates another antiproliferative effect of menin. Both factors are involved in regulation of disruption during oncogenesis apoptotic signal transduction [47-50].

Table 1: PNET-Associated Hereditary Syndromes: Short Description (from [7])

Syndrome	Gene	Patients with PNET (%)
Multiple endocrine neoplasia type I	MEN1 (11q13)	20%–100%
von Hippel–Lindau disease	VHL (3p25–26)	5%–17%
Neurofibromatosis type 1 (Recklinghausen disease)	NF1 (17q11.2)	Rare
Tuberous sclerosis	TSC1 (9q34) / TSC2 (16p13.3)	Very rare

PNET: Pancreatic neuroendocrine tumor.

Antiproliferative menin action is also mediated through inhibition of expression of a human telomerase catalytic component, telomerase reverse transcriptase (hTERT), which has a key role in telomere length support. Hyperexpression of hTERT is directly connected to cell growth and occurs in many oncological diseases [51, 52].

In MEN-1 syndrome, injured cells have weakened DNA reparation ability, which leads to accumulation of point mutations and further chromosomal instability. The role of menin in DNA reparation involves functional interaction with the Fanconi anemia, complementation group D2 protein and replicative protein A type 2 [42, 53, 54].

In some investigations, hyperexpression of PI3K/AKT/mTOR signal pathway genes in MEN-1 syndrome in patients with PNETs has been detected. This is one of the most important associations with

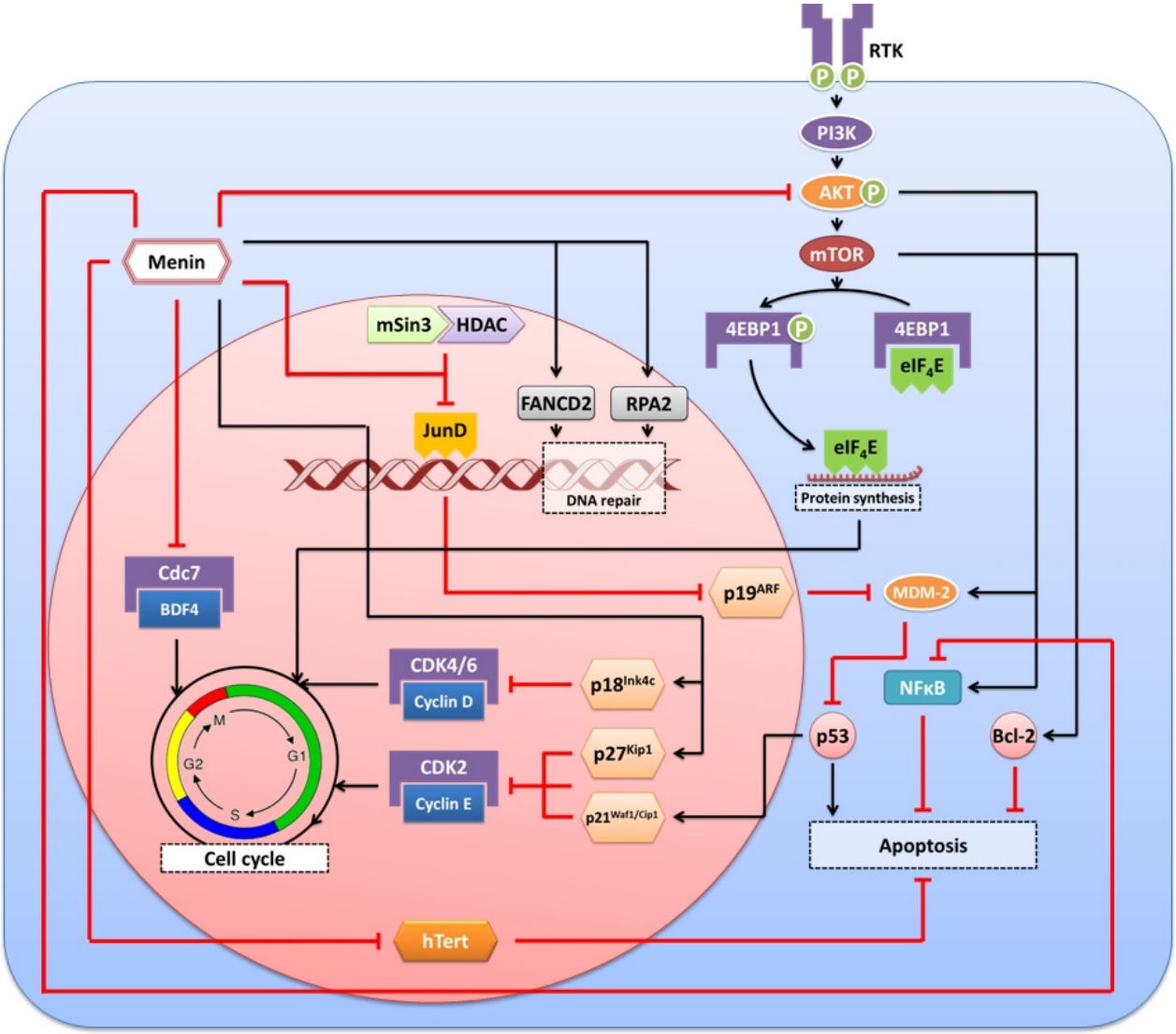

Figure 3: The role of menin as a tumor suppressive protein in intracellular signal transduction. The role of the PI3K/AKT/mTOR signaling pathway in oncogenesis.

Menin regulates the expression of p18INK4C and p27Kip1, both of which are cyclin dependent kinase inhibitors. Another antiproliferative effect of menin is mediated by the inhibition of the transcriptional activity of nuclear factor κB and transcription factor JunD (in the latter case, with the mSin-histone deacetylase complex). Additionally, menin's antiproliferative activity is mediated through inhibition of telomerase reverse transcriptase (hTERT) expression, which has a key role in telomere length support. The role of menin in DNA repair involves its functional interaction with the FANCD2 protein and RPA2.

Note: RTK–receptor tyrosine kinases, P–phosphorylation, PI3K–phosphoinositide 3-kinase, AKT–protein kinase B, mTOR - mammalian target of rapamycin, eIF4E–eukaryotic translation initiation factor 4E, NFκB–nuclear factor κB, MLL–mixed lineage leukemia protein, hTERT–human telomerase reverse transcriptase, HDAC–histone deacetylases, JunD–transcription factor JunD, RPA2–replication protein A2, FANCD2–Fanconi anemia, complementation group D2 protein, CDK2–cyclin-dependent kinase 2, CDK4–cyclin-dependent kinase 4, CDK6–cyclin-dependent kinase 6.

oncogenesis signal transduction pathways [7]. The mTOR protein is a serin/threonine protein kinase, and its activation follows phosphorylation of the 4EBP1 protein and loss of its repressor connection to eukaryotic translation initiation factor 4E (eIF_4E). Unbonded eIF_4E initiates mRNA translation and ribosomal protein synthesis essential for cell proliferation and cell cycle regulation [55-57]. As reported by Kasajima *et al*. [58] and Di Florio *et al*. [59], hyperexpression and activity of mTOR in patients with PNET can have a direct correlation with the presence of distant metastases and are independent risk factors for negative prognosis.

However, the mechanism of PI3K/AKT/mTOR signaling pathway hyperactivation in MEN-1 syndrome in patients with PNET has been less studied. At present, it is supposed that the mechanism involves an inhibitory role of menin toward one of the direct participants, AKT, in this signaling pathway [60].

All of the above-mentioned menin functions and the role of the PI3K/AKT/mTOR signaling pathway in oncogenesis are illustrated in Figure **3**.

With an incidence of about 1:50000, von Hippel–Lindau disease (VHL) is a rare autosomal dominant phacomatosis. VHL is characterized by hypervascular tumors that develop in different organs [33, 61]. The disease is caused by mutations in the *VHL* gene, which encodes the pVHL protein, which in turn is involved in assembly of ubiquitin complex (E3 ubiquitin ligase) and has a role in adaptation to hypoxia. In normoxia, this complex ubiquitinates and causes degradation of hypoxia inducible factor 1α (HIF1α). In hypoxia, pVHL does not bind to HIF1α and causes an increase in HIF1α intracellular content that indirectly leads to hyperexpression of genes encoding vascular endothelial growth factor and platelet-derived growth factor. In normoxia, the mutations, inactivating *VHL* gene, cause HIF1α accumulation followed by expression of the proangiogenic factors mentioned above, proliferation of tumor cells, and angiogenesis [62, 63]. The prognosis in PNET patients with VHL syndrome is more positive than that in patients with sporadic tumors [7]. Although *VHL* mutations in sporadic PNETs are quite rare [7], Schmitt *et al*. [64] found that inactivating mutations in patients with sporadic tumors were definitely associated with lower recurrence-free survival.

Neurofibromatosis type 1 (NF-1), formerly known as von Recklinghausen disease, is an autosomal-dominant gene disorder characterized by flat pigmented lesions, called café au lait spots, and by neurofibromas [33, 65]. PNETs are rarer in NF-1 than in MEN-1 and VHL. The basis of this pathology is the mutation of the *NF1* gene that encodes neurofibromin protein, which is involved in inactivation of various promoting proteins that control dynamic cell growth, particularly the RAS protein and mTOR [7, 66, 67].

Tuberous sclerosis (TS) is another autosomal-dominant hereditary disease associated with PNET, with a population incidence of about 1:10000 [68, 69]. It is interesting to note that in TS, PNETs are quite rare and typically are represented by nonfunctioning tumors. TS development is defined by mutations in the *TSC1* and *TSC2* genes, which encode hamartin and tuberin, respectively [7]. These proteins have a key role in PI3K/AKT/mTOR signaling pathway regulation [70].

Consequently, mutations inactivating the *INK4a* and *MEN1* tumor suppressive genes, mutations of *ATRX/DAXX,* hyperactivation of the PI3K/AKT/mTOR signaling pathway, and increased expression of various growth factors and their receptors could be attributed to basic alterations in signal transduction pathways in PNET cells. Data from molecular investigations are of particular importance for understanding how various targeted drugs directly or indirectly affect the key molecules involved in the described alterations [5, 10, 11, 71, 72].

TARGETED THERAPY OF ADVANCED PNETs

As noted above, the prognosis for patients with advanced and non-resectable PNET forms is negative [4]. Until 2011, the only recommended medication for inoperable patients with PNETs was streptozotocin, an alkylating agent that was permitted for use in 1984 [73]. Furthermore, at present, the efficacy of this medication is a matter of dispute [74]. On the basis of the results of two phase III clinical trials, the targeted medications everolimus and sunitinib became available in 2011 for treatment of advanced PNET forms [10, 72, 73]. Everolimus is a selective mTOR inhibitor. Under the RADIANT-3 protocol, everolimus or placebo were administered to patients with advanced PNETs. The results showed statistically significant improvement of 10 months in the progression-free survival median without tumor progression, which represented a significant increase from the 4.6 months observed in the placebo group ($p < 0.001$). Everolimus therapy was well tolerated by the patients; the most common side effects were stomatitis (7%) and common weakness

(7%) [75]. Similar efficacy results have been demonstrated in a study that compared placebo and sunitinib, which is a multitargeted drug that targets various receptors with tyrosine kinase activity. In patients who were administered sunitinib, the progression-free survival median was 11.4 months, which was a significant improvement over the 5.5 months observed in the placebo group ($p < 0.001$). Furthermore, patient recruitment was terminated early because of the clear demonstration of efficacy. Sunitinib administration was also associated with survival rate increase. By the end of the study, only 9 deaths had occurred in the treatment group compared with 21 deaths in the placebo group ($p = 0.0204$). The survival rate median in the sunitinib group was 30.5 months *vs.* 24.4 months in the placebo group, but the difference was not statistically significant ($p = 0.1926$). The most common side effects of sunitinib therapy were neutropenia (12%), arterial hypertension (9.6%), and bullous pemphigoid (6%) [76].

These therapeutic results stress the significance of PI3K/AKT/mTOR signaling pathway alterations and hyperexpression of growth factors and their receptors in PNET formation. Everolimus inhibits the mTOR protein, which is a key component of the PI3K/AKT/mTOR signaling pathway. Sunitinib inhibits multiple receptors associated with tyrosine kinase activity, thus blocking intracellular signal transduction pathways, including the PI3K/AKT/mTOR pathway [72, 73]. As a result, the inactivation of the PI3K/AKT/mTOR cascade inhibits the growth and proliferation of tumor cells.

Currently, given the importance of the PI3K/AKT/mTOR signaling pathway in the oncogenesis of PNETs, drugs that affect this pathway represent promising therapeutic strategies. As such, the development of PI3K inhibitors, second generation mTOR inhibitors (ATP-competitive mTORC1/mTORC2 dual inhibitors), and drugs with a dual mechanism of action (mTOR/PI3K dual inhibitors) is in progress [71, 77]. In general, these compounds have shown promising early results in preclinical studies. However, randomized trials are required to assess their true potential in the treatment of patients with PNETs.

CONCLUSION

It is important to note that significant progress in the understanding of the molecular basis of PNETs has been made over the last decades. At present, some targeted medications have been approved for use (everolimus, sunitinib), which have provided new perspectives for the treatment of these difficult syndromes, and dozens of promising molecules are currently undergoing clinical trials. Further decoding of the molecular aspects of PNETs pathogenesis will provide new directions for research on potentially more effective methods of treatment that may eventually be integrated into clinical practice.

CONTRIBUTIONS

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Igor V. Maev contributed to the conception; Dmitry N. Andreev and Yuriy A. Kucheryavyy wrote the paper; Diana T. Dicheva provided supportive contributions.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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