Journal of Pathology
J Pathol February 2022; **256:** 143–148
Published online 18 December 2021 in Wiley Online Library (wileyonlinelibrary.com) DOI: 10.1002/path.5834

REVIEW

Clinical implications of cell-of-origin epigenetic characteristics in non-functional pancreatic neuroendocrine tumors

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Abstract

Primary non-functional pancreatic neuroendocrine tumors (NF-PanNETs) are a heterogeneous group of neuroendocrine neoplasms that display highly variable clinical behavior. Therefore, NF-PanNETs often present clinical teams with a dilemma: the uncertain metastatic potential of the tumor has to be weighed against the morbidity associated with surgical resection. Thus, rather than utilizing current radiologic thresholds, there is an urgent need for improved prognostic biomarkers. Recent studies aimed at understanding the epigenetic underpinnings of NF-PanNETs have led to the identification of tumor subgroups based on histone modification and DNA methylation patterns. These molecular profiles tend to resemble the cellular origins of PanNETs. Subsequent retrospective analyses have demonstrated that these molecular signatures are of prognostic value and, importantly, may be useful in the preoperative setting. These studies have highlighted that sporadic NF-PanNETs displaying biomarkers associated with disease progression and poor prognosis, such as alternative lengthening of telomeres, inactivating alpha thalassemia/mental retardation X-linked (*ATRX*) or death domain-associated protein (*DAXX*) gene mutations, or copy number variations, more often display alpha cell characteristics. Conversely, NF-PanNETs with beta cell characteristics often lack these unfavorable biomarkers. Alternative lengthening of telomeres, transcription factor protein expression, and possibly DNA methylation can be assessed in endoscopic ultrasound-guided tumor biopsies. Prospective studies focusing on cell-of-origin and epigenetic profile-driven decision making prior to surgery are likely to be routinely implemented into clinical practice in the near future.

Keywords: non-functional pancreatic neuroendocrine tumors; prognostic markers; epigenetics; ARX; PDX1; ATRX; DAXX; ALT; MEN1

Received 1 September 2021; Accepted 3 November 2021

No conflicts of interest were declared.

Introduction

Pancreatic neuroendocrine tumors (PanNETs) are relatively rare neoplasms, although the incidence in recent years has risen due to increased abdominal imaging [1]. Although functional (i.e. hormone-producing) PanNETs present at an early stage due to tumor-related symptoms and complications, non-functional PanNETs (NF-PanNETs) often remain undetected for years. In addition to sporadic tumors, PanNETs frequently occur in inherited endocrine tumor syndromes, such as multiple endocrine neoplasia type 1 (MEN1) and Von Hippel–Lindau disease [2,3].

The optimal treatment strategy for primary NF-PanNETs is controversial. The clinical dilemma lies in weighing the potential harm from metastatic disease against the complications of surgery. On the one hand, surgical removal of a solitary primary PanNET may prevent local and distant PanNET recurrence, which is relevant as patients with localized disease have a reported 5-year survival of 93% compared with 27% of patients with metastatic disease [1]. However, primary NF-PanNET surgical resection does not completely eliminate the risk of distant metastases; in a large recent retrospective series, 25% of NF-PanNET patients developed recurrent disease after surgical intervention [4]. On the other hand, pancreatic surgery carries significant risk of complications, such as pancreatic fistulas and delayed gastric emptying, in addition to endocrine and exocrine pancreatic insufficiency [5].

It is well established that the risk of metastatic disease is associated with increased tumor size [4]. Consequently, current guidelines recommend to surgically resect NF-PanNETs >2 cm in diameter and, optionally, to closely monitor patients with NF-PanNETs <2 cm [6,7]. The World Health Organization proliferation-based grading system is currently the best guideline for the prediction of metastatic potential after surgery [8]. Other predictive factors include perineural or lymphovascular invasion [9]. However, these parameters can only be reliably established after resection of the primary tumor. Therefore, preoperative clinical biomarkers that can be used to better predict aggressive behavior of primary PanNETs prior to any intervention are urgently needed.

Understanding the molecular underpinnings of this disease is essential for improving strategies to improve accurate diagnoses and subsequent treatment modalities. Exome sequencing studies have revealed that the DNA mutational spectrum in NF-PanNETs predominantly includes mutations in genes encoding epigenetic regulators, such as *MEN1*, alpha thalassemia/mental retardation X-linked (*ATRX*), and death domain-associated protein (*DAXX*) [10,11]. A multistep genetic process appears to be a prerequisite for PanNET initiation and progression [12,13]. *ATRX* and *DAXX* gene inactivation and subsequent alternative lengthening of telomeres (ALT) phenotype activation are associated with increased metastatic potential in sporadic primary PanNETs, but have not been included in clinical decision making to date [4,14–18].

Chemical modifications of DNA or histones (e.g. histone acetylation and DNA methylation) regulate global gene transcription that can directly control cell proliferation and tissue differentiation. Disruption of these epigenetic mechanisms can result in hyperplasia and neoplasia. Recent studies have eloquently demonstrated that genome-wide DNA methylation or histone modification signatures are linked to intrinsic cellular states and tumor cell origins in NF-PanNETs. Here, we provide an overview of the recent literature with regard to epigenetic characteristics of NF-PanNETs, with a focus on cell-of-origin aspects, genetics, and genome-wide DNA methylation and histone modification analyses. The future opportunities for translation to clinical practice are highlighted.

Cell differentiation in the development of the endocrine pancreas

The pancreas is an endoderm-derived organ. The exocrine and endocrine pancreas develop from common progenitor cells through organized patterns of gene transcriptional regulation and extracellular signals, such as Notch and WNT signaling. Although many of the insights into pancreatic endocrine development are derived from studies in mice, several key concepts appear to be shared with humans (reviewed in [19]). Pancreatic and duodenal homeobox 1 (PDX1) is an essential transcription factor for early pancreatic development [20]. Multipotent progenitor cells differentiate into exocrine cells, as well as the endocrine alpha, beta, delta, epsilon, and pancreatic polypeptide cell lineages. These individual endocrine cell lineages each arise from neurogenin 3 (NEUROG3)-expressing precursor cells [21]. Subsequent temporal waves of transcription factor expression result in terminal endocrine differentiation. Whereas neurogenic differentiation factor 1 and aristaless-related homeobox (ARX) are required for the glucagon-producing alpha cell fate, NK2 homeobox 2 and paired box 4 (PAX4) act to induce the insulin-producing beta cell lineage [22,23]. Notably, PDX1 is essential for beta cell identity maintenance [24]. Alterations in gene expression of these transcriptional regulators can lead to alpha cell to beta cell transdifferentiation and vice versa, highlighting the plasticity of these epigenetic cell differentiation programs [25,26]. The bZIP transcription factors, MAFA and MAFB, have been shown to play an important role in the functional maturation of alpha and beta cells [27,28]. From this cell lineage development paradigm, glucagonomas are considered to originate from alpha cells and insulinomas from beta cells. Until recently, the endocrine lineage associated with NF-PanNETs was unknown.

The genetic basis of epigenetic characteristics of NF-PanNETs

In 2011, Jiao *et al* [10] reported recurrent somatic mutations in the *MEN1* and *ATRX/DAXX* genes in a series of sporadic NF-PanNETs. These findings were later confirmed and expanded using whole-genome sequencing [11]. *MEN1*, *ATRX*, and *DAXX* are tumor suppressor genes and their functional loss appears to be critical in a large proportion of NF-PanNETs.

The *MEN1* gene (located on chromosome 11q13), which is also mutated in the inherited form of the disease, MEN1 syndrome, encodes the menin protein that is involved in transcriptional regulation by connecting transcription factors to chromatin modifying enzymes (reviewed in [29]). The role of menin as a scaffold protein in mixed-lineage leukemia 1/2 (MLL1/2; KMT2A/B) containing histone H3 lysine 4 trimethylation (H3K4me3, a mark of active gene transcription) methyltransferase complexes has been described and investigated extensively [30,31]. Loss of menin appears to be an early event in sporadic NF-PanNET development (Figure 1) [12]. However, the exact mechanism of PanNET tumorigenesis driven by loss of *MEN1* is unknown. Silencing of *MEN1* does not appear to result in global chromatin alterations, but rather subtle changes to the core transcriptional regulatory circuitry [32]. Genetic rescue of H3K4me3 loss in a MEN1 mouse model resulted in reduced PanNET-related death [33]. Whereas somatic *MEN1* mutations are found almost exclusively in NF-PanNETs and only rarely in sporadic insulinomas,

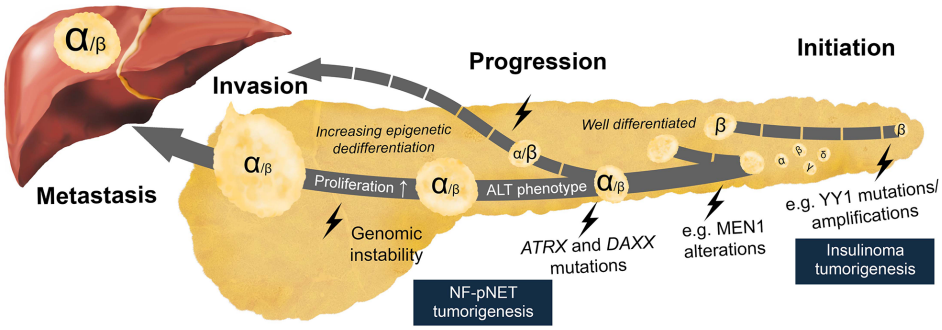

Figure 1. Multistep tumorigenesis of sporadic NF-PanNETs. Inactivation of *MEN1*, *ATRX/DAXX*, and genomic instability is associated with the highest recurrence rate and liver metastases after NF-PanNET surgical resection. This combination of genetic events occurs predominantly in alpha cell-like/intermediate-type lesions. An absence of *ARTX/DAXX*-inactivating mutations reduces metastatic potential in alpha cell-like NF-PanNETs. Most NF-PanNETs of the beta cell-like subgroup have an indolent course. Functional primary insulinomas follow a tumorigenic route that is distinct from NF-PanNETs, characterized by mutations in *YY1* and/or other gene amplification events.

MEN1 patients frequently develop insulinomas and, in *Men1* mouse models, insulinomas are common, a seemingly contradictory finding [34,35].

Mutations in *ATRX* (located on chromosome Xq21.1) and *DAXX* (chromosome 6p21) are found in about a third of sporadic NF-PanNETs [11]. The exact mechanism of how functional loss of these proteins contributes to NF-PanNET development is unknown. However, ATRX and DAXX form a histone chaperone complex that functions to deposit the histone variant H3.3 into repetitive heterochromatin (e.g. pericentromeric and telomeric regions) characterized by histone H3 lysine 9 trimethylation (H3K9me3, a mark of inactive chromatin) [36–38]. The presence of inactivating *ATRX/DAXX* mutations is strongly associated with the presence of ALT, a telomerase-independent telomere maintenance mechanism. Subsequently, ALT has been used as a surrogate biomarker for functional loss of the ATRX/DAXX complex and is a causal factor for cancer cell immortalization [14]. By facilitating the replication of G4-DNA structures, ATRX protects cells from accumulation of genomic instability. Thus, loss of ATRX increases chromosomal instability and mutational burden [39,40]. As ATRX and DAXX are also present at gene promoters, disruption of global transcriptional regulation due to loss of ATRX/DAXX potentially contributes to NF-PanNET tumorigenesis [38,41]. Highlighting the importance of cellular context, within all neuroendocrine neoplasms, *ATRX/DAXX* mutations and ALT are found almost exclusively in PanNETs and are not present in other cellular origins [4]. Similar to *MEN1* loss, *ATRX/DAXX* mutations or protein loss are only rarely found in insulinomas, with the exception of a subgroup of NF-PanNETs that appear to have acquired insulin production at an advanced stage [9]. ATRX/DAXX protein loss is not the initiating genomic alteration, but rather occurs at a later stage in the development of the primary NF-PanNET that is associated with progression to metastatic disease [12,13,16] (Figure 1). These observations are consistent with the positive correlation between the presence of ALT with increasing tumor size, as well as the findings of subclonal ATRX/DAXX protein loss in primary PanNETs [16,17]. Moreover, patients with the ATRX syndrome do not develop NETs [42]. Importantly, ALT and loss of DAXX/ATRX protein in primary PanNET is a strong prognostic biomarker of recurrence and/or development of metachronous metastatic disease [4,15,16,43].

Epigenetic signatures of NF-PanNETs

In contrast to many other tumor types, sporadic NF-PanNETs harbor relatively low numbers of DNA mutations. However, as the recurrent mutations occur in genes involved in epigenetic processes, several research groups have recently focused on global DNA and histone modification signatures in NF-PanNETs. Based on observations that intrinsic cellular identities are defined by enhancer signatures, chromatin immunoprecipitation of histone H3 lysine 27 acetylation (H3K27ac, a mark of active enhancers) combined with next generation sequencing was performed in a series of NETs, including NF-PanNETs [44]. Hierarchical clustering revealed two major NF-PanNET enhancer profile subtypes: beta cell-like tumors and alpha cell-like tumors. In addition, smaller groups of mixed and signature negative tumors were identified. The transcription factors PDX1 and ARX were found to be useful surrogate immunohistochemical markers to distinguish these tumor subtypes. Interestingly, ALT was predominantly associated with the alpha cell-like subtype. As PanNETs lacked active *NEUROG3* and *PAX4* loci, and enhancer activity was low at terminal differentiation genes (e.g. *MAFA* and *FFAR1*), the authors concluded that ARX and PDX1 expressing NF-PanNETs partially resemble mature alpha and beta cells, respectively, rather than progenitor cells. In a retrospective study of 83 sporadic and MEN1-associated NF-PanNETs, the immunohistochemical absence of PDX1 was independently associated with increased postoperative liver metastases. Similarly, a recent study found that *PDX1* methylation was associated with worse prognosis; however, this

analysis included both functional and NF-PanNETs, which may have influenced the findings, as insulinomas have a favorable prognosis and are usually not methylated at the *PDX1* promoter [45]. However, a recent comprehensive international retrospective follow-up study in a cohort that included 561 sporadic NF-PanNETs did not identify ARX or PDX1 status as an independent prognostic predictor for relapse-free survival [4]. In this study, ALT status (and loss of ATRX/DAXX protein) was associated with ARX protein and a lack of PDX1 protein, and demonstrated an independent prognostic significance. Therefore, ARX-positive NF-PanNETs may represent the subtype that has the highest metastatic potential, probably due to its susceptibility for ALT activation. Additionally, tumors expressing both ARX and PDX1 may represent an intermediate subtype.

Indeed, in a series of 64 functional and NF-PanNETs, Chan *et al* [46] showed that *ATRX/DAXX* and *MEN1* mutated PanNETs exhibited DNA methylation and gene expression signatures related to alpha cells and displayed high ARX protein levels, whereas PDX1 was suppressed due to *PDX1* promoter methylation. This so-called A-D-M mutant subgroup (or MAD+, as described by others) showed decreased recurrence-free survival compared with PanNETs without this mutational profile. In a later study of 125 non-functional and sporadic PanNETs with regard to *ATRX/DAXX* and *MEN1* mutational status and whole genome DNA methylation profiles, PanNETs were stratified into three subgroups according to their proximity to alpha and beta cells in phyloepigenetic analyses [47]. These subgroups included an alpha cell-like subgroup characterized by *MEN1* gene mutations, a beta cell-like subgroup with few genetic alterations, and an intermediate subgroup with *ATRX/DAXX* and *MEN1* mutations in 67% of tumors and more frequent copy number variations. The absence of *ATRX/DAXX* and *MEN1* mutations in the beta cell-like subgroup is in line with reports that functional primary insulinomas do not harbor these gene mutations, but rather mutations in *YY1* or other epigenetic modifier genes [34] (Figure 1). Interestingly, intermediate tumors were associated with the shortest disease-free survival and resembled alpha cells rather than beta cells, as 86% expressed ARX by immunostaining. These findings were confirmed in an independent study of 84 sporadic functional and NF-PanNETs [48]. The majority of copy number variations were found in the subgroup with a high prevalence of *ATRX/DAXX* and *MEN1* mutations. These subgroups did not show differential global DNA methylation levels, but could be categorized based on differences in methylation of gene loci, including O6-methylguanidine-methyltransferase (*MGMT*), *PDX1*, and caspase 8 (*CASP8*).

Conclusion and clinical implementation

Several recent studies have contributed to elucidating the molecular mechanism of NF-PanNET tumorigenesis using epigenetic profiling, in some cases combined with mutational profiling. Taken together, these studies revealed the presence of distinct NF-PanNET subgroups based on epigenetic signatures of endocrine differentiation. These epigenetic signatures resemble either mature alpha or beta cells, rather than pancreatic progenitor cells. Although there are limitations in these studies with regard to the inclusion of functional tumors, the retrospective nature of the study designs and the different clinical endpoints, it is clear that epigenetic signatures matter for the behavior of NF-PanNETs and will probably be incorporated into clinical decision making in the near future. In summary, beta cell-like tumors harboring few gene mutations and expressing PDX1 usually have an indolent course. Similarly, alpha cell-like tumors that often carry *MEN1* but no *ATRX/DAXX* mutations also recur infrequently. However, alpha cell-like NF-PanNETs that express ARX, harbor a combination of *ATRX/DAXX* and *MEN1* gene mutations, and display increased copy number variation portend the most unfavorable prognosis (Table 1). In addition, primary functional insulinomas represent a separate tumor entity with a unique genetic and epigenetic background. However, in rare cases, alpha cell-like NF-PanNETs may transdifferentiate by acquiring the ability to secrete insulin, usually presenting with a larger tumor size and aggressive phenotype. In numerous retrospective studies, ALT and/or ATRX/DAXX protein loss has been shown to be the strongest predictor of recurrent disease after surgery.

Although the field is exciting and moving forward rapidly, several issues remain unresolved. For example, why are NF-PanNETs with alpha cell-like characteristics particularly susceptible for inactivating *ATRX/DAXX* mutations and subsequent ALT activation? Moreover, it is unknown if NF-PanNETs with beta cell-like characteristics that acquire an *ATRX* or *DAXX* mutation may then acquire alpha cell-like characteristics. Interestingly, it was reported that ALT is more frequent in NF-PanNETs in men than women, but other risk factors are unknown [4]. Furthermore, the exact tumorigenic mechanism and thus potentially targetable consequences of *ATRX/DAXX* loss remain unclear. Finally, although

Table 1. Cell-of-origin derived subclasses of sporadic NF-PanNETs.

Epigenetic subtype	Alpha cell-like	Alpha cell-like/intermediate	Beta cell-like
MEN1 mutation	+	+	–
ATRX/DAXX mutation	–	+	–
CNV	–	+	–
Recurrence rate	Low	High	Low

CNV, copy number variation.

MEN1 syndrome-related NF-PanNETs have been included in some reports, it is unclear if these observations can be extrapolated directly to MEN1-related and other inherited types of NF-PanNETs.

Endoscopic ultrasound-guided fine needle aspiration/biopsy (EUS FNA/B) is one of the cornerstones of PanNET diagnostics. It has been demonstrated that detection of ALT, ATRX/DAXX, ARX, and PDX1 protein can be carried out reliably on EUS FNA/B [49,50]. It is reasonable to postulate that DNA methylation and copy number variation analysis may also be readily assessed on EUS FNA/B. Another way forward could be to identify epigenetic marks in blood that may predict the clinical behavior of PanNETs, for example with the use of cell-free DNA [51].

Epigenetic signatures of endocrine differentiation offer important opportunities to improve diagnosis and treatment of patients with NF-PanNETs. To selectively treat patients who are at higher risk for metastatic NF-PanNET development and, conversely, to surveil patients who are at lower risk, future prospectively directed studies aimed at assessing cell-of-origin epigenetic signatures as preoperative predictors for developing advanced NF-PanNETs are warranted.

Author contributions statement

All authors were involved in the literature search, generation of figures and writing the paper and had final approval of the submitted and published versions.

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