Molecular pathology and genetics of gastrointestinal neuroendocrine tumours

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Purpose of review
Neuroendocrine tumours (NETs) of the luminal gastrointestinal tract and pancreas are increasing in incidence and prevalence. Prior assumptions about the benign nature of 'carcinoids' and the clinical importance of distinguishing functional vs. nonfunctional tumours are being overturned through greater understanding of disease behaviour and heterogeneity. This review highlights the most contemporary genetic and molecular insights into gastroenteropancreatic NETs.

Recent findings
Biomarkers such as neuron-specific enolase or chromogranin A could be supplemented or supplanted by PCR-based analysis of NET genes detectable in the blood transcriptome. Conventional pathology, including Ki67 testing, could be enhanced with immunohistochemistry and exome analysis. Prognostic markers and/or putative therapeutic targets uncovered through recent studies include heparanase, Id, ATM, SRC, EGFR, hsp90 and PDGFR.

Summary
After a long-standing paucity of options for conventional cytotoxic therapy, the comprehension and treatment of gastroenteropancreatic NETs has been enriched by advancements in taxonomy, molecular pathology and genetic/epigenetic testing.

Video abstract available
See the Video Supplementary Digital Content 1 (http://links.lww.com/COE/A4).

Keywords
gastroenteropancreatic neuroendocrine tumours, gastrointestinal neuroendocrine tumours, molecular disease

INTRODUCTION

The incidence and prevalence of gastrointestinal neuroendocrine tumours (GI NETs, also known as gastro-entero-pancreatic or GEP NETs) are increasing, likely not only through improved pathologic classification of these tumours as well as their incidental discovery via endoscopic and radiographic methods of screening for other cancers, but also through postulated epidemiologic factors such as the widespread use of proton pump inhibitors (PPIs) [1]. The understanding of the pathogenesis of GI NETs is expanding as well, with insights derived from molecular pathology and high-throughput genomic analyses [2]. Deeper comprehension of disease biology has already led to novel therapeutic interventions, such as everolimus targeting alterations in the mammalian target of rapamycin (mTOR) pathway [3] and sunitinib acting as a multitarget tyrosine kinase inhibitor [4].

BIOMARKERS

Serologic testing is frequently used during the diagnosis and longitudinal monitoring of GI NETs, particularly when trying to ascertain treatment response. Chromogranin A (CgA) and neuron-specific enolase (NSE) have been used as general biomarkers for these tumours, and can provide valuable prognostic information. For instance, early

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KEY POINTS

- A novel 51-gene PCR-based assay performed on peripheral blood may prove to have higher sensitivity and specificity than the CgA biomaker.
- The distinction between functional and nonfunctional NETs is archaic, whereas NETs of the pancreas and the luminal digestive tract are biologically and genetically distinct.
- Bcl2, Id1 and heparanase are among the clinically relevant candidates for future targeted therapies.

declines in CgA and NSE after initiation of everolimus in patients with advanced pancreatic NETs have been associated with prolonged progression-free and overall survivals (OS) [5].

However, CgA levels can also be confounded by the use of PPIs, which are often necessarily prescribed to patients with GI NETs who require acid-suppressive therapy, especially those with gastrinomas; discontinuation of PPIs for up to 2 weeks is required to negate the medications' effect upon CgA levels [6]. Other criticisms of CgA testing have included heterogeneity among assays [7], as well as their identification of a secretory peptide that is not strictly linked to tumour proliferation. CgA levels can also often be discordant with disease burden. For example, it can be lower in diffuse metastases than in hepatic metastases alone [8], or not produced at all in up to a quarter of GI NETs [9▪▪]. The utility of CgA as a biomarker varies by how it is used. It is generally not sensitive or specific enough for screening or diagnosis but can be useful for prognostication and longitudinal follow-up of patients.

Rather than performing ELISA for a single peptide, a new PCR-based analysis of 51 marker genes in the blood has been proposed [9▪▪]. The list of genes whose expression is measured – which includes somatostatin receptors 1, 3, 4 and 5 – was assembled rationally through the identification of mutual upregulation between NET tissue microarray datasets and the blood transcriptome, as well as from a literature-curated panel. Because the assay hinges on the circulating transcript signature of NETs, all 51 included genes were validated as having mRNAs that are reliably detectable in the peripheral blood. Both at the time of diagnosis and when measuring responses to therapy, this approach may permit more sensitive and specific testing of GI NETs than CgA. The general applicability of such an approach will require prospective validation.

PATHOLOGY

The pathologic taxonomy of GI NETs is a subject of debate, if not controversy, with important international distinctions [10▪]. Older classification systems hinged on embryology and categorized GI NETs by their site of origin in the foregut, midgut or hindgut. Modern staging systems may account for site and grade, as well as TNM (tumour-node-metastasis) assessments of size, invasiveness and extent of disease [11]. Irrespective of which staging system is used, tumour grade is a key pathological determinant of prognosis, and it is particularly important to dichotomize aggressive, highly proliferative NETs requiring immediate therapeutic intervention from more indolent tumours in which surveillance may be permissible or preferred.

The proliferative rate of a neuroendocrine tumour can be assessed by counting the number of mitoses present in each high-powered field (hpf) under microscopy or by calculating the percentage of cells that stain with antibody for Ki-67, which is only expressed in the S, G2 and M phases of the cell cycle [12]. The Ki-67 proliferative index informs grading in the WHO and European Neuroendocrine Tumor Society (ENETS) systems such that the lowest-grade (G1) tumours must demonstrate both less than two mitoses per 10 hpf and a Ki67 less than 3%. Grade 2 (G2) tumours have Ki67 indices of 3–20%, and grade 3 (G3) tumours have Ki67 indices more than 20%. These demarcations in grading have proven prognostic and predictive utility and thus affect treatment decisions. Although Ki-67 testing may be particularly useful in cases in which limited tissue is available for examination under an adequate number of hpfs, its intraobserver and interobserver variability has also been criticized, especially its ability to consistently distinguish G1 vs. G2 NETs [13]. The appropriate Ki-67 cut-off between G1 and G2 remains a matter of debate; 5% has been proposed as a more discriminatory cutpoint, such that raising the threshold between G1 and G2 to 5% resulted in better differentiation of outcome, including OS [14]. In a multivariate analysis that included lymph node ratio, a Ki-67 of more than 5% has been identified as the strongest predictor of recurrence after resection of malignant pancreatic neuroendocrine tumours [15]. As a predictive marker, tumours with a Ki-67 of more than 55% appear more responsive to platinum-based chemotherapy [16].

Therefore, although clinicopathologic data remain the cornerstones upon which management decisions are made about GI NETs, information on gene expression may go beyond histologic appearance and mitotic index to enable a more nuanced comprehension of tumour behaviour by which treatment plans can be individualized.

NOVEL GENETIC TARGETS

Most contemporary staging systems distinguish well differentiated pancreatic NETs from poorly differentiated neuroendocrine carcinomas (NECs), the latter of which are high-grade by definition [10▪]. Using immunohistochemistry and targeted exomic sequencing to compare NECs against NETs, Yachida *et al.* [17] identified abnormal immunolabelling of p53 and Rb in the majority of the high-grade neuroendocrine carcinomas, whereas the same targets were intact in well differentiated tumours. Overexpression of Bcl-2, an antiapoptotic protein, was also detected in the majority of NECs (100% in the small cell variant), vs. a minority of NETs, and correlated significantly with higher mitotic rate, suggesting Bcl-2 antagonism as a possible mechanism of action for treating these high-grade neuroendocrine carcinomas [17].

Poorly differentiated pancreatic neuroendocrine neoplasms were further studied in a group of RIP1-Tag2 mice, in which a viral oncogene, SV40 T-antigen, was used to inactivate the p53 and Rb tumour suppressor pathways, thereby inducing tumourigenesis in pancreatic islets [18]. Although the majority of tumours generated in this model were insulinomas, histologic analysis was used to isolate a novel class of tumours with a high nuclear: cytoplasmic ratio, anaplastic morphology and invasion into the surrounding exocrine pancreas; this subset of tumours was then tested and found to have a loss of markers of beta-cell differentiation, including insulin. The Id1 gene (inhibitor of differentiation/inhibitor of DNA binding), previously isolated from cancer stem cells [19], was specifically expressed by the same tumours. The implication is that patients with poorly differentiated neoplasms, typically treated systemically with platinum-based chemotherapy, may also be candidates for treatments that target the Id family [18].

Another newly identified marker of invasiveness has been heparanase, whose enzymatic activity allows NETs to degrade their extracellular matrix and penetrate through endothelium. Within the aforementioned RIP1-Tag2 model, high heparanase expression correlated with higher grade, a more advanced stage, and a proclivity for metastasis. Increased peritumoural lymphangiogenesis via heparanase upregulation provided a potential route for dissemination, whereas heparanase deletion increased pericyte coverage of endothelial cells [20], making heparanase itself another attractive target for future therapy.

Comparison of gene expression signatures in metastatic vs. nonmetastatic NETs revealed that the Ataxia Telangiectasia Mutated (*ATM*) gene was negatively correlated with metastatic behaviour. In addition, strong correlations were seen between high levels of ATM mRNA, immunohistochemical positivity for the ATM protein and low Ki-67 indices. Immunohistochemical negativity for ATM (seen in over 85% of the metastatic samples) was associated with significantly decreased OS in comparison to patients with ATM positivity (respective median OS: 2.7 years vs. not reached, $P = 0.003$). Therefore, ATM downregulation has been proposed as a promoter of metastasis, putatively through a loss of its regulatory function in the cell cycle [21]. Given evidence from other tumour types, such as lymphoma [22], that poly[ADP-ribose] polymerase (PARP) inhibitors impede the growth of ATM-null or ATM-mutant cancer cells by enabling intolerable DNA damage to accumulate, there is preclinical rationale for testing PARP inhibitors in ATM-negative NETs.

Other possible gene targets were unveiled by the largest whole-exome sequencing to date of GI NETs [23], in which 48 small intestine NETs underwent massively parallel exome sequencing, revealing alterations in genes responsible for chromatin remodelling, apoptosis and RAS signalling, among other mechanisms of oncogenesis. A host of therapeutically relevant alterations, including SRC, EGFR, hsp90 and PDGFR, were found [23].

CHROMOSOMAL ALTERATIONS BY ANATOMIC SITE

The mutations that lead to neuroendocrine tumourigenesis may occur either at the level of alleles and/or at the chromosomal level, where instability can lead to loss or gain of chromosomal segments or even whole chromosomes (aneuploidy). Comparative genomic hybridization (CGH) tends to show, overall, more losses than gains in NETs [24], and loss of chromosome 18 may be particularly divergent among anatomic subsites of NETs. An MD Anderson study found that allelic loss of chromosome 18 was present in 69% of ileal carcinoid tumours, vs. 13% of nonileal carcinoid tumours and 6% of pancreatic neuroendocrine tumours ($P = 0.001$). All the ileal tumours with loss of chromosome 18 had complete loss of both chromosomal arms, which was significantly associated with smaller tumour size, although the same tumours showed a higher tendency toward hepatic metastasis than tumours at nonileal sites [25]. Compounding long-standing concern that Oberndorfer's nomenclature for carcinoids may lead to the mistaken presumption of uniformly benign behaviour among these tumours, such discoveries underscore that neuroendocrine tumours of the luminal digestive tract should be considered biologically distinct from pancreatic NETs.

The importance of chromosome 18 has since been validated in a separate genome-wide association study [26] in which constitutional genetic polymorphisms in chr18q22.1 were found to be more common in patients with ileal carcinoids vs. controls, highlighting the pathogenic significance of this particular deletion.

Genome-wide single-nucleotide polymorphism comparisons among ileal, nonileal and pancreatic NETs not only revealed loss of chromosome 18 as the most frequent allelic imbalance but also identified loss of part or all of chromosome 21 as significantly associated with nonileal site and larger size of tumour. Loss of chromosome 13 or 13q, chromosome 16 or 16q, and amplification of chromosome 20 or 20p were, in descending order, the next most frequent imbalances among all well differentiated NETs [27].

Changes in the copy number of chromosome 3p are also frequent events in advanced pancreatic NETs. An analysis of 113 primary pancreatic NETs (the majority well differentiated) found a 20% rate of 3p alteration, with losses found in well differentiated tumours but never in poorly differentiated carcinomas, of which half of the small sample size ($n = 6$) actually showed gain of 3p. The study also included 32 neuroendocrine metastases, in which monosomy for 3p was significantly more common than in primary tumours [28].

FUNCTIONAL VS. NONFUNCTIONAL NEUROENDOCRINE TUMOURS

Although it is important to recognize the differences in nomenclature and biology between luminal carcinoids and pancreatic NETs, elsewhere in the classification schema of neuroendocrine neoplasms, it has been suggested that the long-held distinction between functional and nonfunctional tumours be abandoned. NETs have been defined as functional if they release bioactive peptides that cause symptoms, for example flushing, bronchospasm, and diarrhoea within the carcinoid syndrome, and the clinical burden of hormonal secretion often served as the trigger for intervention upon the culprit tumours. However, because functional and nonfunctional NETs are otherwise inseparable in terms of histopathology and immunohistochemistry, there is concern that this distinction is arbitrary and may lead to undertreatment of nonfunctional tumours, especially as many intestinal NETs may only become symptomatic after hepatic metastasis. Among pancreatic NETs, nonfunctional tumours actually have a worse prognosis than insulinomas, and equivalent survival to the other functional pancreatic NETs (e.g. glucagonomas, somatostatinomas, gastrinomas). Later stage at diagnosis, perhaps due precisely to the lack of overt functionality, may contribute to the reduced life expectancy of patients with nonfunctional NETs [29]. The gastro-entero-pancreatic tissues that can give rise to NETs are heterogeneous, especially in the islets of Langerhans in which alpha, beta and delta cells produce glucagon, insulin and somatostatin, respectively; so, it is not surprising that neuroendocrine neoplasms arising from the same organs and with otherwise identical tumour biology can have different clinical phenotypes.

The progression of pancreatic beta-cells from hyperinsulinism to benign insulinomas to malignant carcinomas has been studied from novel angles, as morphologic predictors of disease course are lacking and, for the most part, insulinomas have been considered to carry a superior prognosis; in fact, their inclusion among the functional pancreatic NETs likely inflates the survival estimates for that entire category [29]. The islets of Langerhans ordinarily stain strongly for NOTCH1, but it appears that insulinomas lose their immunohistochemical positivity for NOTCH1 as they evolve. In a retrospective study [30] of human disease specimens, NOTCH expression was seen in 56% of 32 well differentiated insulinomas but was completely absent in 15 carcinomas. In a similar immunohistochemical comparison of benign and malignant insulinomas, EpCAM, a morphoregulatory molecule in the islets of Langerhans that is known to be overexpressed in progenitor cells during foetal development, appeared to be reactivated and overexpressed (assessed by 3+staining) in malignant insulinomas and their metastases significantly more often than in benign insulinomas [31].

Mutations of the glucagon receptor in the alpha cells have been implicated in the newly discovered Mahvash disease, in which homozygous inactivation of the *GCGR* gene encoding the glucagon receptor leads to hyperglucagonemia, alpha-cell hyperplasia and pancreatic NETs [32,33]. The hereditability of the disease has yet to be established.

MENIN: A SPORADIC AND GERMLINE MUTATION

Indeed, although the majority of pancreatic NETs occur sporadically, hereditary predispositions do exist in the forms of von HippelLindau (vHL) syndrome, neurofibromatosis type 1 (NF 1), tuberous sclerosis and multiple endocrine neoplasia type 1 (MEN 1) [34]. In the latter, the germline mutation of menin on chromosome 11 leads to pancreatic NETs, in addition to pituitary adenomas and hyperparathyroidism. In accordance with Knudsen's two-hit hypothesis, loss of heterozygosity at 11q13 is

characteristic of patients with germline MEN1 mutations, and the 'second hit' appears to arrive via negative inhibition of menin by the oncogenic microRNA (oncomir)miR-24-1, suggesting a potential therapeutic application for a silencing RNA antagomir in restoring menin's onco-suppressive role [35].

However, although MEN 1 remains rare, affecting an estimated 1 in 30 000 individuals [36], somatic MEN1 mutations are much more common and are seen in up to 40% of sporadic pancreatic NETs, and is also the most common somatic mutation identified in lung carcinoids [37]. The epigenetic understanding of menin was recently furthered through the elaboration of its link to the Hedgehog pathway. Menin directly interacts with protein arginine methyltransferase 5 (PRMT5) and recruits PRMT5 to methylate, a repressive site in the promoter of the Gas1 gene, a crucial factor for the binding of the Sonic Hedgehog ligand to its receptor. After ablation of menin, and in the absence of its negative regulatory role, the pro-proliferative Hedgehog signalling pathway becomes activated, leading to tumourigenesis. Important preclinical work by Gurung *et al.* [38▪] has already shown in MEN1-excised mice harbouring insulinomas that treatment with a Hedgehog inhibitor results in decreased islet cell proliferation. This discovery unveiled the link between menin and the Hedgehog pathway, thus revealing a new therapeutic target for tumours whose growth is driven by mutations in menin [38▪], especially as Hedgehog inhibitors are already commercially available for the treatment of basal cell carcinoma and medulloblastoma.

CONCLUSION

The pathologic criteria for classifying GI NETs have been refined over time to reflect a deeper understanding of differences in differentiation and anatomic site. The genetic fingerprint of NETs is detectable through sophisticated serology that can be used by the clinician at diagnosis and during longitudinal care. Treatment decisions should no longer be based on the perception of tumour functionality, but can be informed by advances in understanding of disease biology and genetics, which increasingly reveal candidates for targeted therapy.

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Conflicts of interest

There are no conflicts of interest.

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- ▪ of special interest
- ▪▪ of outstanding interest

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