Hallmarks of gastrointestinal neuroendocrine tumours: implications for treatment

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Abstract

In the past few years, there have been advances in the treatment of neuroendocrine tumours (NETs) and improvements in our understanding of NET biology. However, the benefits to patients have been relatively modest and much remains yet to be done. The 'Hallmarks of Cancer', as defined by Hanahan and Weinberg, provide a conceptual framework for understanding the aberrations that underlie tumorigenesis and to help identify potential targets for therapy. In this study, our objective is to review the major molecular characteristics of NETs, based on the recently modified 'Hallmarks of Cancer', and highlight areas that require further research.

Key Words

- cancer
- neuroendocrine tumours
- treatment
- tumorigenesis

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Introduction

Neuroendocrine tumours (NETs) are diverse in their site of origin and clinical behaviour, ranging from highly aggressive small cell cancers of the lung to indolent, low-grade tumours of the small bowel (Yao *et al.* 2008a). Symptoms arise from both the tumour burden and the secretion of bioactive hormones by the so-called 'functional tumours'. High-grade tumours are often treated with chemotherapy, while for low-grade tumours, the focus may be on alleviating the consequences of

hormone secretion with somatostatin analogues (SSAs; Ramage *et al.* 2012). The recent approval of sunitinib (Raymond *et al.* 2011) and everolimus (Yao *et al.* 2011) for the treatment of pancreatic NETs provides evidence for the importance of angiogenesis and the mTOR pathway in the pathogenesis of NETs, but there remains a significant unmet need to improve outcomes in this disease. Recently, Hanahan and Weinberg have published an updated version of their original 'Hallmarks of Cancer', in

Figure 2

Hallmarks of neuroendocrine tumours: mechanisms of tumourigenesis and targets for therapy. BEZ, BEZ235 or NVP-BEZ235, an imidazoquinoline derivative; CTLA4, cytotoxic T-lymphocyte antigen 4; ERK1/2, extracellular signal-regulated protein kinase 1/2; GPCR, G-protein coupled receptor; HIF1 α , hypoxia-inducible factor-1 α ; MEK, mitogen-activated protein kinase

kinase; mTOR, mammalian target of rapamycin; C1, mTOR complex 1; C2, mTOR complex 2; PD1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; PI3K, phosphatidylinositol 3-kinase; SHC, Src homology 2 domain containing.

excellent targets for gastrointestinal NET (GEP-NET) diagnosis and therapy (Pavel 2013).

Somatostatin receptors

The most widely studied are the somatostatin receptors (SSTRs), which bind somatostatin and its therapeutic analogues. Five SSTR subtypes (SST1–SST5) have been identified. Two spliced variants of *SSTR2* (*SSTR2A* and *SSTR2B*) have been found, with *SSTR2A* having the highest expression and the greatest relevance from a biological and clinical perspective. Somatostatin and its synthetic analogues (octreotide and lanreotide) act through the five specific SSTRs found on the cell membranes of various tumours, including GEP-NET (Diakatou et al. 2011). The first step of the signalling cascade following agonist stimulation is the internalisation of the SSTR (Scott et al. 2002). Functional coupling of receptors is part of the

signalling process, with dimerisation occurring between different SSTR subtypes (homodimerisation) or between SSTRs and other GPCRs (heterodimerisation) (Duran-Prado et al. 2008). SSAs preferentially target the *SSTR2*, which is highly expressed in GEP-NETs. However, some tumours are resistant to SSAs and it is not known whether the defect lies in the activation of the SSTR or downstream signalling events. Phosphorylated *SSTR2*s are present in most GEP-NETs from patients treated with octreotide, but their sub-cellular distribution varies markedly (Waser et al. 2012a). The activation of SSTRs, particularly *SSTR2* and *SSTR5*, can block the secretion of biologically active peptides from tumour cells (Cakir et al. 2010a,b, Gatto & Hofland 2011) and may lead to the inhibition of cell growth and the induction of apoptosis (Strosberg & Kvolis 2010).

The internalisation of activated SSTRs together with their bound ligands allows cold or radiolabelled

somatostatin/SSAs to be administered (Cescato *et al.* 2006), with obvious implications for the radiological diagnosis and treatment of GEP-NETs. Hence, SSAs are the standard treatments for GEP-NETs, in particular those associated with carcinoid syndrome (ENETS 2008, Gatto & Hofland 2011). Evidence obtained recently indicates that SSAs have an anti-proliferative effect on GEP-NETs via a number of pathways (Strosberg & Kvolts 2010). Both octreotide and lanreotide, which have slightly different binding affinities to SSTR2 and SSTR5 (the most clinically relevant SSTR subtypes), can exert an *in vivo* anti-proliferative role. Results from a phase III randomised placebo-controlled trial have indicated that long-acting octreotide can significantly prolong the time to tumour progression in patients with a metastatic midgut NET (Rinke *et al.* 2009) and this has recently been confirmed for intestinal and pancreatic NET patients using lanreotide autogel in the CLARINET study (Caplin *et al.* 2014). New SSAs such as pasireotide, which binds four of the five SSTR subtypes with a high affinity, have been developed to optimise the activation of SSTRs expressed at GEP-NET cell membranes. In a phase II study, pasireotide appeared to be reasonably well tolerated and effective in controlling carcinoid syndrome in patients with an octreotide-refractory or -resistant advanced tumour (Kvolts *et al.* 2012). However, pasireotide does not appear to be superior to the other commercially available SSAs in controlling the symptoms of carcinoid syndrome (Wolin *et al.* 2013).

The functional interaction between SSTR subtypes or with other GPCRs, such as the dopamine receptors, can exert a functional effect on the signalling cascade and the strength of the response to the agonist. As SSTRs and dopamine D2 receptors can be co-expressed in GEP-NET cells, it has been proposed that some interaction between the complex formed by the activated receptor and the β -arrestin of the two receptor families could affect the downstream signalling (Gatto & Hofland 2011).

Other GPCRs

Dopamine, a well-characterised neurotransmitter, acts through five dopamine receptors (D1–D5) that are expressed in endocrine tumours (Diakatou *et al.* 2011). The D2 receptor is the most relevant from a clinical perspective. Using RT-PCR, *DRD2* mRNA has been found in all samples of a series of 35 GEP-NETs, although the expression level was generally lower than that in other NETs (O'Toole *et al.* 2006). The presence of D2 receptors has also been evaluated by immunohistochemistry in well-differentiated NETs originating from different sites.

Receptor expression was found in 85% of the examined tumours, with particularly high expression observed in pancreatic and duodenal NETs (Grossrubatscher *et al.* 2008). Targeting the D2 receptor might be an effective mechanism for suppressing NET cell hormone secretion (Gatto & Hofland 2011). As the activation of these receptors induces phosphodiesterase activity and decreases intracellular cAMP concentration, the consequent inhibition of MAPK signalling might inhibit cell proliferation. Interestingly, an unfavourable clinical outcome has been observed more frequently among GEP-NET patients with low D2 immunoreactivity, although no significant correlation between D2 receptor expression and Ki-67 expression was reported (Grossrubatscher *et al.* 2008). Thus, the clinical utility of targeting the dopamine receptor in GEP-NETs remains unclear.

The GLP1 receptor has been shown to be greatly over-expressed in NETs, particularly in insulinomas, hence it may represent a novel molecular target for *in vivo* scintigraphy and targeted radiotherapy. For GLP1 receptor scintigraphy, a low background signal can be expected due to low receptor expression in normal tissues surrounding the tumours (Korner *et al.* 2007). GLP1 receptor imaging is an innovative, non-invasive, diagnostic approach that successfully localises small insulinomas pre- and intra-operatively (Christ *et al.* 2009). As opposed to benign insulinomas, malignant insulinomas often lack GLP1 receptors, but frequently express SSTR2s which can be targeted (Wild *et al.* 2011).

Glucose-dependent insulintropic polypeptide (GIP) receptors are expressed in the majority of pancreatic, small bowel and bronchial NETs. Receptor binding and mRNA analysis by PCR reveal GIP receptors in many GEP-NETs (Waser *et al.* 2012b). Indeed, most SSTR-negative NETs and GLP1-receptor-negative malignant insulinomas are GIP-receptor-positive. As most epithelial and stromal gastrointestinal tumours and lung adenocarcinomas are GIP-receptor-negative, the specificity of this receptor could aid GEP-NET targeting (Waser *et al.* 2012b).

The secretin receptor is also expressed in some NET cell types. This receptor mediates the effects of the gastrointestinal hormone, secretin, on digestion and water homeostasis via the phosphatidylinositol 3 kinase (PI3K)/serine–threonine protein kinase pathway. *In vitro*, tumour cells with high secretin receptor expression respond well to PI3K inhibitors, depending on the quantitative receptor expression, and hence this may represent a new pathway to explore in some GEP-NET types (Lee *et al.* 2012).

It is well known that serotonin (5-HT), an amine secreted by many NETs, is a strong inducer of fibrosis, although the mechanisms involved in fibrosis and proliferation within tumours are not fully determined (Svejda *et al.* 2010). The fibrotic process often has an influence on the clinical outcome for GEP-NET patients in terms of development and management of mechanical symptoms. Targeting the 5-HT receptors might allow new anti-proliferative and anti-fibrotic strategies for small bowel NETs by inhibiting the activity of fibroblasts and NET cells in the tumour microenvironment (Svejda *et al.* 2010).

The cause of neuroendocrine hyperplasia as found in the ileum (Sherman *et al.* 1979, Moyana & Satkunam 1992) and pancreas (Anlauf *et al.* 2007) is in most cases not known. However, in the stomach (Solcia *et al.* 1988), the process is partially understood: hypergastrinaemia stimulates continuous proliferative signalling in enterochromaffin-like cells. Results of research on both model systems and patients have indicated the gastrin/CCK2 receptor to be a potential target for future anti-tumour therapy. The CCK2 receptor is expressed in cancers, where it contributes to tumour progression and is over-expressed in a sub-set of tumours, allowing for its use in tumour targeting with a radiolabelled ligand (Froberg *et al.* 2009, Sanchez *et al.* 2012). Antagonising CCK2 receptors inhibits gastrin-mediated enterochromaffin-like cell secretion and proliferation, thus inhibiting *in vivo* tumour development in rodents (Martinsen *et al.* 2003) as well as in patients with gastric carcinoids (Fossmark *et al.* 2012).

A newly discovered GPCR is the olfactory receptor 51E1 which is significantly expressed in different types of NETs (Cui *et al.* 2013, Giandomenico *et al.* 2013).

Evading growth suppression

An important characteristic of cancer cells is their ability to circumvent cellular growth suppression programmes, most of which are dependent on the activity of tumour suppressor genes.

Several tumour suppressor genes are involved in the pathogenesis of GEP-NETs through evasion of growth suppression. Germline-inactivating mutations of the tumour suppressor gene *MEN1* are responsible for the majority of familial NETs (Capurso *et al.* 2006) and somatic mutations of this gene are present in approximately one-third of sporadic foregut NETs (Rindi & Bordi 2005). PNETs also occur in von Hippel–Lindau (VHL) disease, which is caused by a mutation in the *VHL* tumour suppressor gene (Capurso *et al.* 2006).

Neurofibromatosis type 1 (NF1) and tuberous sclerosis (TS) are both inherited syndromes that can rarely develop PNETs. NF1 and TS are caused by inactivating mutations of the tumour suppressor genes *NF1* (17q11.2) and *TSC1* (9q34) plus *TSC2* (16p13.3) respectively. All these genes code for proteins involved in the negative regulation of the PI3K–AKT–mTOR pathway – a key pathway in the control of cell survival and proliferation (Rindi & Bordi 2005, Capurso *et al.* 2006). Altered upstream regulators of mTOR (phosphatase and tensin homologue (PTEN) and *TSC2*) have also been found in sporadic PNETs (Krausch *et al.* 2011, Cingarlini *et al.* 2012).

The retinoblastoma tumour suppressor pathway is inactivated in the majority of PNETs (Tang *et al.* 2012). One of the mechanisms by which the retinoblastoma pathway inactivation occurs is through over-expression of cyclin-dependent kinases CDK4 or CDK6. These results provide a strong rationale for the use of inhibitors of CDK4/CDK6 as a potential therapy in PNETs (Tang *et al.* 2012).

The dysfunction of the p53 pathway is another important mechanism contributing to the initiation and progression of PNETs (Hu *et al.* 2010, Yachida *et al.* 2012). This gene plays a crucial role in the control of apoptosis, the cell cycle, genomic stability and inhibition of angiogenesis. Although *p53* (*TP53*) mutations are rare in NETs, the p53 pathway is commonly altered in PNETs through aberrant activation of its negative regulators (MDM2, MDM4 and WIP1) (Hu *et al.* 2010). Reactivation of p53 through inhibition of these negative regulators is a potential therapeutic strategy for PNETs (Lehmann & Pietenpol 2012). Somatic mutations targeting the cell cycle regulator gene *CDKN1B* have recently been detected in 8% of small intestine NETs (Francis *et al.* 2013).

The down-regulation of other tumour suppressor genes (*RASSF1A*, *CDKN2A*, *HIC1* and *MGMT*) by promoter hypermethylation has been reported to be an important event in NET development (Chan *et al.* 2003, House *et al.* 2003, Liu *et al.* 2005, Zhang *et al.* 2006, Arnold *et al.* 2007), indicating a potential use of epigenetic therapy in NETs (Rahman *et al.* 2010).

Avoiding immune destruction

Research in cancer immunotherapy is partially based on the concept that cancer cells express antigens that elicit T cell-mediated responses. Patients have been vaccinated against different tumour antigens in attempts to direct patients' own immune systems to attack cancer. However, successful immune system responses against tumours are frequently hindered by the tumour microenvironment,

preventing the immune system from eradicating malignant cells (Hanahan & Weinberg 2011).

In the last decade, development of immune-competent genetically modified mice has allowed the role of the immune system to be studied, and thereby the elucidation of the complex mechanisms that drive this immune escape (Abe & Macian 2013). NET cells may escape the immune response by several mechanisms. Tumour-associated antigens (TAAs), along with cytokines from NET cells, dendritic cells (DCs) and tumour-associated macrophages, recruit/induce regulatory T cells (Tregs), which inhibit the anti-tumour immune response (Ameri & Ferone 2012). Altered expression of HLA class I molecules has been demonstrated in ten out of 11 pancreatic NETs, loss of β 2-microglobulin being the most frequent alteration (Ryschich et al. 2003). Immunohistochemical analysis of four patients with lung NET revealed the complete absence of cells expressing DC markers CD1a and CD83, and induction of apoptosis in DCs is a key mechanism by which tumours escape immune recognition and elimination (Katsenelson et al. 2001).

Tregs are crucial for maintaining peripheral tolerance against self-antigens but have also been demonstrated to hinder successful immunotherapy (Zou 2006). In an exploratory study of 68 patients with GE-NETs, increased frequencies of circulating Tregs were demonstrated and, compared with those of healthy donors, a decreased proliferative capacity of T cells and reduced levels of Th1-promoting cytokines were observed (Vikman et al. 2009). This means that therapeutic strategies are needed to overcome tumour-induced immune suppression. This need has led to the successful development of antibodies directed against cytotoxic T-lymphocyte-associated antigen 4 (CTLA4), programmed cell death protein 1 (PD1) and its ligand (PDL1) (Brahmer et al. 2012, Topalian et al. 2012). The use of Ipilimumab, a fully human MAB that blocks CTLA4 and promotes anti-tumour immunity, has resulted in improved survival in patients with metastatic melanoma (Hodi et al. 2010), but there are no studies reported on NETs.

Enabling replicative immortality

Telomerase could be an appropriate molecular target for cancer treatment as 90% of human cancers show telomerase activity (Orlando & Gelmini 2001), and reconstruction of longer telomeres as a result of telomerase activity could contribute to the unlimited replicative capacity of cancer cells. Telomerase activity can be inhibited in several ways including small-molecule inhibitors, antisense

oligonucleotides, immunotherapies and gene therapies (Ruden & Puri 2013). Some new therapies targeting telomerase activity have entered phase I and II clinical trials, for indications other than GEP-NETs.

Telomerase activity has been suggested as a marker of malignancy in NETs. In patients with pulmonary NETs, telomerase activity was normal in typical carcinoids but elevated in atypical carcinoids, large-cell neuroendocrine cancer and small-cell lung cancer (Gomez-Roman et al. 2000, Zaffaroni et al. 2005, Nishio et al. 2007). Data on telomerase activity in GEP-NET patients are very limited: results from one study in patients with pancreatic NETs (including nine insulinoma patients) indicated that telomerase activity could be a tool for determining the malignant potential of these tumours (Lam et al. 2000), whereas results of another study in a heterogeneous population did not reveal a clear association between telomerase activity and malignancy (Bockhorn et al. 2000).

Exomic sequencing of pancreatic NETs has revealed mutations in genes encoding either of the two subunits of a transcription/chromatin remodelling complex consisting of death-domain-associated protein (DAXX) and alpha thalassaemia/mental retardation syndrome X-linked (ATRX) in 43% of cases (Jiao et al. 2011). Inactivating mutations in the ATRX or DAXX genes are strongly correlated with the typical features of the telomerase-independent telomere maintenance mechanism termed 'alternative lengthening of telomeres' (ALT) (Heaphy et al. 2011). Therefore, targeting ALT could be another therapeutic approach for those patients with pancreatic NETs, either alone or in combination with targeting telomerase (Shay et al. 2012).

On the basis of the results of a very recent study, loss of DAXX or ATRX protein and ALTs were concluded to be associated with chromosome instability and shorter survival times in patients with pancreatic NETs (Marinoni et al. 2014). In contrast, ALT probably is not an appropriate target in patients with small bowel NETs as exome sequencing did not detect mutations in the DAXX or ATRX genes in these tumours (Banck et al. 2013).

Tumour-promoting inflammation

Research over the past decade has revealed, paradoxically, that the inflammatory response enhances tumourigenesis and progression (Hanahan & Weinberg 2011). Interferon (IFN) was introduced in 1982 to treat patients with metastatic mid-gut NETs and carcinoid syndrome (Oberg et al. 1983). Possible mechanisms are the inhibition of cell proliferation, immune cell-mediated cytotoxicity,

inhibition of angiogenesis and reduction in tumour growth by blocking the cell cycle (Oberg 1992). Immune cells present at the tumour site were therefore thought to be an attempt by the immune system to eradicate tumours, but are increasingly acknowledged to play a role in tumour progression. For example, activation of nuclear factor κ B (NF κ B) by the classical inhibitor-of-NF κ B kinase β (IKK β)-dependent pathway is a crucial mediator of inflammation-induced tumour growth and progression, as well as an important modulator of tumour surveillance and rejection (Karin & Greten 2005). A case-control cohort study, including 50 GEP-NET patients, detected a difference in the insertion/deletion genotype of the NF κ B1-94 ATTG promoter between patients with pNETs and those with carcinoid tumours (Burnik & Yalcin 2009).

Several pro-inflammatory cytokines play a role in the development of NETs. Results from a study comparing pNET patients and healthy unrelated controls indicated an association between the high-expression C/T-511 *IL1 β* (*IL1B*) genotype and susceptibility to pNETs (Cigrovski Berkovic et al. 2012). Interestingly, macrophage migration inhibitory factor (MIF) has been recently linked to neuroendocrine differentiation in prostate cancer (Tawadros et al. 2013). Immunotherapies are now being used clinically to inhibit several immune cell subsets that promote tumour development (Coussens et al. 2013).

In NETs, oncolytic viruses are being explored for their capacity to alter the immunosuppressive tumour microenvironment and activate immune effector cells (Essand et al. 2011). The oncolytic adenovirus Ad5 (CgA-E1A-miR122) has been shown to selectively replicate and kill neuroendocrine cells, including freshly isolated midgut carcinoid cells from liver metastases (Leja et al. 2011). The highly immunosuppressive microenvironment enables the oncolytic virus to replicate. This leads to a microbe-associated inflammatory response, which is optimal for antigen presentation by DCs. In addition, TAAs released from the dying tumour cells can be captured by the DCs. In order to enhance this effect even further, strategies have been developed to arm oncolytic viruses with immune-stimulatory genes (Essand 2013). A phase I trial (NCT00314925) is being planned to study the side effects and the best dose of Seneca Valley virus-001 for the treatment of patients with advanced solid tumours with neuroendocrine features.

Activating invasion and metastasis

For the establishment of distant metastasis, loss of cellular adhesion, invasiveness, intravasation, extravasation and

proliferation in the host organ are required. Loss of E-cadherin expression and function has been associated with disruption of E-cadherin junctions and gain of cell motility and invasiveness in several tumour types. Moreover, E-cadherin loss was identified in 13 out of 17 (76.5%) gastric neuroendocrine carcinomas (NECs) and was significantly associated with lymph node metastasis; however, it did not correlate with invasion to adjacent organs or distant metastasis (Boo et al. 2007). Additionally, progressive loss of E-cadherin in tumour cells with nuclear β -catenin accumulation indicates that they have undergone an epithelial-mesenchymal transition – a developmental regulatory programme resulting in invasion, resistance to apoptosis and dissemination (Hanahan & Weinberg 2011).

In one study cytoplasmic/nuclear β -catenin staining was observed in 79% of GI-NETs, and the genetic analysis showed a mutant β -catenin (S37A) in 37% of tumours. This mutation prevents its ubiquitination and prolongs its half-life compared with WT β -catenin. The authors did not find mutations in the other cases with positive β -catenin staining (nuclear/cytoplasmic), and presumably, its accumulation is due to other molecular alterations not yet identified (Fujimori et al. 2001). In another study it was observed that in those patients with GEP-NETs showing high SNAIL1 (SNAI1) protein levels, a cytoplasmic E-cadherin pattern, reduced N-cadherin expression and loss of E-cadherin/ β -catenin adhesion complex integrity at the cell membrane, the 5-year survival rate was reduced (Galvan et al. 2013). Another important factor in the tumour invasion and metastasis process is the matrix metalloproteinase (MMP)/tissue inhibitor of metalloproteinase (TIMP) system. There are differences in the expression of MMP/TIMP between benign and malignant lesions (Jeffery et al. 2009), and MMP activity can be detected in cancer cells before the detection of extra-vascular metastasis (Zhang et al. 2010). The expression of MMP2 in pancreatic NETs (particularly in gastrinomas) could be used to characterise a malignant phenotype, whereas weak expression of MMP9 could indicate a less invasive phenotype (Gurevich 2003).

Src family kinases (SFK) are non-receptor tyrosine kinases that respond to mitogenic stimuli to interact with proteins involved in cell adhesion, motility and spreading. Up-regulation of SFK in pancreatic endocrine tumours has been confirmed, expression being higher in primary lesions than in metastases (Capurso et al. 2006, Di Florio et al. 2007). Moreover, the activation of SFK during cell adhesion stimulates the mTOR pathway and leads to increased cell cycle protein synthesis (Di Florio et al. 2011).

The inhibition of SFK by PP2 in cultured pancreatic cells caused a delay in cell adhesion and impaired the migration and colonisation process (Di Florio *et al.* 2007). Additionally, the inhibition of SFK prevents the up-regulation of the mTOR escape pathways usually triggered by mTOR inhibitors (Di Florio *et al.* 2011). The effects of Src inhibition have been studied on neuroendocrine cancer stem cells (N-CSCs), marked inhibition of tumour growth was observed (Gaur *et al.* 2011). These findings indicate potential new therapy strategies using mTOR and Src inhibitors together to reduce proliferation, cell adhesion and spreading of tumour cells.

Inducing angiogenesis

During tumour progression, the so-called 'angiogenic switch' is activated that promotes angiogenesis required for tumour growth (Hanahan & Folkman 1996). Vascular endothelial growth factor (VEGF) is a key pro-angiogenic cytokine and crucial for NET carcinogenesis and progression. Patients with NETs have higher levels of circulating VEGF than control subjects, and those with progressive disease have higher levels than those with stable disease (Pavel *et al.* 2005, Zhang *et al.* 2007). There are conflicting results regarding whether pancreatic or midgut NETs have higher levels of VEGF (Terris *et al.* 1998, Bello *et al.* 2006). Low-grade NETs have extraordinary vascularisation, but it is diminished in more aggressive tumours (Marion-Audibert *et al.* 2003). In islet cell tumours, higher microvascular density (MVD) and VEGF (VEGFA) expression predict a more favourable prognosis (Couvelard *et al.* 2005) and this has been called the 'neuroendocrine paradox' (Scoazec 2013).

Treatment with a neutralising antibody to VEGF in mice with a duodenal carcinoid resulted in decreased tumour size and inhibition of liver metastasis (Konno *et al.* 1998). Furthermore, a MAB that blocks the VEGFA ligand and an antibody blocking the VEGF receptor subtype 2 have been tested in the RIP-Tag2 mouse model of insulinoma with consistent antiangiogenic effects on microvessel density, endothelial cell proliferation and anti-tumour activity with increased apoptosis (Casanovas *et al.* 2005, Sennino *et al.* 2012). The humanised anti-VEGF MAB, bevacizumab, reduced VEGF expression, MVD and tumour growth in a human carcinoid xenograft model, but does not affect the growth of carcinoid cells *in vitro* (Zhang *et al.* 2007).

Bevacizumab has been studied in patients with well-differentiated NETs in a phase II trial in combination with octreotide, achieving an 18% response rate (Yao *et al.* 2008b). A phase II study in combination with

2-methoxyestradiol revealed some degree of tumour reduction without achieving objective response according to RECIST response criteria (Kulke *et al.* 2011). Bevacizumab has also been studied in phase II trials in NETs in combination with temozolomide, with higher response rates observed in pancreatic NET G2 (Chan *et al.* 2012, Koumarianou *et al.* 2012) and in combination with oxaliplatin and capecitabine, reaching an objective response rate of 23% (Kunz *et al.* 2010). A randomised comparison of bevacizumab and octreotide vs IFN and octreotide is ongoing (NCT00569127).

Other proangiogenic growth factors are also important in tumour growth. Serum angiopoietin 2 (ANG2) and TIE2 levels are significantly elevated in patients with NETs compared with controls, and in patients with distant metastases compared with those without metastasis. Time to disease progression is shorter in patients with higher serum ANG2 levels (Srirajskanthan *et al.* 2009, Detjen *et al.* 2010, Figueroa-Vega *et al.* 2010). Inhibitors of the TIE2 pathway are being evaluated in other solid tumours and may be worth exploring for NETs.

In human pNET samples, platelet-derived growth factor receptors α (PDGFR α (PDGFRA)) and β (PDGFR β (PDGFRB)) are commonly expressed both in tumour cells and tumour stroma (Fjallskog *et al.* 2003). Experimental studies with the RIP-Tag2 transgenic mouse model have demonstrated that dual blockage of endothelial cells and pericytes with VEGFR and PDGFR small molecule inhibitors results in a significant synergy (Pietras & Hanahan 2005). Sunitinib (an inhibitor of both PDGFR and VEGFR) is approved for the treatment of G1 and G2 pNETs, after its use in a phase III trial resulted in an increase in patient progression-free survival (Raymond *et al.* 2011). It has also been tested in NETs from other locations (Kulke *et al.* 2008), and other tyrosine kinase receptor inhibitors such as pazopanib are currently being studied (Ahn *et al.* 2013). However, the emergence of resistance to antiangiogenic therapy remains a major clinical challenge, but may be reduced by the concurrent inhibition of c-Met (Sennino *et al.* 2012) or the combination of bevacizumab and tyrosine kinase inhibitors (Castellano *et al.* 2013).

Other angiogenesis inhibitors such as angiostatin and endostatin have been studied in the past and, although they have shown antiangiogenic and antitumour effects in various mouse models (Hanahan 1985, Bergers *et al.* 1999), these findings did not translate to humans in a phase II trial of endostatin in NETs (Kulke *et al.* 2006a). However, there are indirect antiangiogenic mechanisms that may provide a therapeutic target, such as the inhibition of the PI3K-AKT-mTOR pathway and the effect of SSAs.

IFN also seems to have an antiangiogenic effect. In patients who have received IFN α treatment, liver metastasis biopsy material shows lower VEGF plasma levels and reduced VEGF mRNA levels and MVD (von Marschall *et al.* 2003). Thalidomide has been studied in two phase II trials in NETs, not reaching partial responses in monotherapy (Varker *et al.* 2008), but demonstrating promising activity in combination with temozolomide (Kulke *et al.* 2006b), although the mechanism is unclear.

Another important factor involved in angiogenesis is the hypoxia-inducible factor (HIF). In a series of 86 GEP-NETs, expression of HIF1 α (HIF1A) as measured by immunohistochemistry has proved in multivariate analysis to be a predictor of OS (Pinato *et al.* 2014). In another series of 24 ileal carcinoids, HIF1 α and HIF2 α were highly expressed. The HIF2 α expression was higher in metastases compared with the primary tumour in the same patient (Arvidsson *et al.* 2010). Heterozygous mutations in the *SDH* (*SDHB*) gene activate the angiogenic pathway mediated by HIF1 α and VEGF, while *SDHD* mutations may be involved in tumourigenesis of midgut carcinoids (Kytola *et al.* 2002).

Genome instability and mutation

Genome instability and mutation are considered to be basic characteristics of tumours that enable multistep progression, to which defects in caretaker mechanisms, mutations of genes involved in DNA repair mechanisms, chromatin remodelling and cell cycle control may all contribute. Genome instability can be best analysed by three molecular genetic approaches studying: i) microsatellite instability; ii) chromosomal instability and iii) methylation patterns including the CpG island methylator phenotype (CIMP) (Arnold *et al.* 2008). Poorly differentiated and aggressive metastatic tumours harbour more chromosomal alterations than their more differentiated counterparts. Methylation abnormalities including global hypomethylation of the genome (Karpathakis *et al.* 2013) and hypermethylation of tumour suppressor gene promoters leading to their inactivation have been described for a wide array of human malignancies (Robertson 2005).

Similar to other tumour entities, allelic imbalances are more frequent in poorly differentiated GI-NETs than in well-differentiated tumours (Furlan *et al.* 2004). Moreover, frequent *TP53* alterations have also been described in these tumours (Lubensky & Zhuang 2007). The chromosomal (comparative genome hybridisation) profiles of fore- and midgut NETs are different (Tonnie *et al.* 2001). When comparing fore/midgut NETs with poorly

differentiated colorectal NETs, significant differences in microsatellite instability have not been detected. But CIMP was more abundant in poorly differentiated colorectal NETs (Arnold *et al.* 2008). Microsatellite instability appears to be an infrequent event in intestinal NETs (Kidd *et al.* 2005, Arnold *et al.* 2007, 2008). CIMP-negative NETs of the foregut and midgut had a better clinical outcome than their CIMP-positive counterparts and this finding has also been associated with Ki-67 positivity (Arnold *et al.* 2007). Hypermethylation of the promoters of RAS association 148 domain gene family 1 (*RASSF1*) and cyclin-dependent kinase inhibitor 2a/p16INK4a tumour suppressor genes has been noticed both in pancreatic and other GI-NETs (Karpathakis *et al.* 2013).

Benign insulinomas have been associated with an increased methylation rate (Arnold *et al.* 2007). Hypermethylation of the differentially methylated region 2 (DMR2) regulating the expression of the insulin-like growth factor 2 (IGF2)/H19 locus and over-expression of IGF2 has been described in insulinoma (Dejeux *et al.* 2009). IGF2 over-expression may contribute to growth of insulinomas similar to other tumours, such as adrenocortical cancer (Bertherat & Bertagna 2009).

Differences between pancreatic and small intestinal NETs have been observed at several levels. In contrast to small intestinal NETs, high levels of microsatellite instability have been reported in two studies of pNET, and this has been associated with the inactivation of the mutL homologue 1 (*MLH1*) gene (Mei *et al.* 2009). Methylation profiles of NETs of pancreatic and intestinal origin are also different (Chan *et al.* 2003). Novel studies using the next-generation sequencing approaches underline the relevance of somatic mutations in both small intestinal and pancreatic NETs, but the spectra of mutations are different. In small intestinal NETs, frame-shift mutations and hemizygous deletions of the cell cycle regulator cyclin-dependent kinase inhibitor 1B, alternatively p27 (*CDKN1B*) gene have been found (Francis *et al.* 2013). In contrast to intestinal NETs, pancreatic NETs are more likely to be associated with rare hereditary tumour syndromes including multiple endocrine neoplasia type 1 (MEN1), VHL disease and tuberous sclerosis (TSC). Somatic mutations of the *MEN1* gene have been found in 44% of PNET samples (Jiao *et al.* 2011). The protein product of the tumour suppressor *MEN1* gene, menin, interacts with mixed lineage leukaemia (MLL (KMT2A)) proteins, having histone-modulating methyltransferase activity (Yokoyama *et al.* 2004). As noted earlier, somatic mutations in the *DAXX* and *ATRX* genes that are also pivotal in the regulation of chromatin remodelling have

been noted in 43% of pNET cases, but these are absent from intestinal NETs (Jiao *et al.* 2011). Somatic mutations of *TSC2* and *PTEN* tumour suppressor genes involved in the mTOR pathway have also been noted in 14% of pNETs (Jiao *et al.* 2011). All these observations are indicative of major differences in the molecular pathogenesis of pancreatic and intestinal NETs.

In vitro experiments on cell lines have the potential to produce novel therapeutic options (Larsson 2013). Epigenetic mechanisms might be of interest, as they are potentially reversible (Karpathakis *et al.* 2013). Inhibition of DNA methyltransferases, for example with azacytidine or via histone deacetylation (valproic acid, sodium butyrate, MS-275 (a synthetic benzamide) and trichostatin A), seems to be effective in GEP-NET cell lines; however, there are no results regarding clinical applications as yet (Larsson 2013).

Resisting cell death

Evasion of cell death is one of the hallmarks of human cancers, which promotes tumour formation and progression as well as treatment resistance.

Several tumour suppressor genes and oncogenes involved in the pathogenesis of GEP-NETs have a major role in the modulation of cell death. Most of these genetic and epigenetic alterations over-activate Akt–mTOR signalling (Ciuffreda *et al.* 2010) and promote resistance to cell death. In contrast, mTOR hyper-activation represents an important target for cancer therapy. Everolimus is an oral mTOR inhibitor that has been extensively studied for NETs (Yao *et al.* 2008b, 2011, Pavel *et al.* 2011, Faggiano *et al.* 2012). However, the emergence of drug resistance may limit the utility of mTOR inhibitors (Carew *et al.* 2011). mTOR signalling consists of two distinct complexes: mTORC1 and mTORC2. In NET cells, everolimus blocks mTORC1 activity but does not alter the mTORC2 complex at nanomolar concentrations. Thus, it is possible that inhibition of mTORC1 shifts the balance to increased mTORC2 activity, which has been shown to activate Akt directly (Sarbasov *et al.* 2005) or through up-regulation of IGF1 signalling (Tamburini *et al.* 2008). These results provide a strong rationale for dual targeting of mTORC1 and PI3K activity (Zitzmann *et al.* 2010).

Another potential approach to reverse resistance to mTOR inhibitors is the use of SSAs through suppression of PI3K–Akt signalling (Bousquet *et al.* 2012). Significant clinical improvement has recently been observed after treatment with everolimus and octreotide LAR in NET patients (Pavel *et al.* 2011) and the tolerability of

everolimus combined with pasireotide is currently under investigation (NCT00804336).

Dysregulating cellular energetics

In normal cells, energy is mainly produced by oxidative phosphorylation of glucose, but in cancer cells energy metabolism is characterised by increased glycolysis and lactate production, even under aerobic conditions, known as the Warburg effect. The amount of ATP production is much lower in glycolysis compared with oxidative phosphorylation, therefore cancer cells compensate for this lower efficiency by increasing glucose uptake – this can be visualised using fludeoxyglucose positron emission tomography (FDG-PET). In NETs, FDG-PET positivity is associated with a high proliferation rate and poor prognosis (Binderup *et al.* 2010).

In recent years, there has been renewed interest in metformin, a drug that has been used for decades to treat type II diabetes but may have anticancer properties. Metformin usage has been associated with better survival in diabetic pancreatic cancer patients (Sadeghi *et al.* 2012) and with a greater likelihood of complete pathological tumour response following neo-adjuvant chemotherapy in diabetic breast cancer patients. Metformin reduces insulin resistance in patients with type II diabetes and thus lowers levels of insulin and IGFs, which may contribute to its anti-cancer effects. Metformin also activates AMPK, which results in the inhibition of mTOR signalling (Jalving *et al.* 2010). Thus, the combination of metformin and everolimus or temsirolimus could result in synergistic tumour growth inhibition and abrogate the hyperglycaemia caused by mTOR inhibitors. Results of a phase I trial of metformin and temsirolimus in advanced cancer have been published (MacKenzie *et al.* 2012), with further ongoing clinical trials of the mTOR inhibitor/metformin combination in advanced cancer. However, there are no clinical trials of metformin as a monotherapy or in combination therapy in patients with GEP-NETs.

Conclusions and perspectives

The pre-clinical and clinical results reviewed in this study not only support the notion that the ‘hallmarks of cancer’ are relevant to NETs, but also highlight the fact that little research has been done in certain areas that may be of potential therapeutic value.

As in other cancers, allelic imbalances, *TP53* alterations, CIMP and increased telomerase activity are more frequent in poorly differentiated GI-NETs than in

well-differentiated tumours; however, microsatellite instability appears to be an infrequent event in intestinal NETs. Whole-genome sequencing of both germline and tumour DNA is expected to generate more precise data about the molecular pathogenesis of endocrine cancers.

Sustained proliferation signalling plays an important role in tumourigenesis, from hyperplasia to neoplasia, at least in gastric NET types 1 and 2. However, hyperplastic cells have been found at the periphery of NETs in the appendix and intestine, indicating a similar process of tumourigenesis but the proliferation signalling pathways in these tumours are not yet fully elucidated.

It has been demonstrated that blocking the gastrin-mediated proliferation signal may lead to complete tumour regression in gastric NETs, and the results of phase II and phase III studies with blockers of EGFR (erlotinib and gefitinib)-mediated proliferation pathways and with dual-blockers of PI3K-Akt/mTOR pathways are expected to define its usefulness in treating NETs.

Progress has also been made in the exploration of signalling pathways mediated by GCPR, with interesting results for the development of diagnostic tools such as scintigraphy with SST or GLP1 analogues, and therapeutic strategies either by selective agonism of SSTRs or blocking CCK2 receptors, for example.

Another hallmark of cancer demonstrated in NETs is the capability to evade growth control mechanisms mediated by tumour suppressor genes such as *VHL*, *TSC*, *NF1*, *MEN1* and *RB* among others. Based on preclinical experiments, results indicate the potential of gene therapy for the induction of expression of suppressor genes, such as menin in NET patients who are carriers of inactivating mutations.

Additionally, results of recent experiments have confirmed the ability of NET cells to evade control by the immune system and their ability to induce inflammation to promote greater infiltration. Studies are being performed with immunomodulatory molecules and modified T cells to stimulate the immune system in order to induce regression in NETs.

Regarding invasion and metastasis, results from recent studies have challenged the established clonal evolution theory of tumourigenesis, and different models have been proposed to explain the early metastatic outgrowth of less immunogenic tumour cells without the mutations of the primary tumour cells, with the possibility of the early migration of a subset of stem-like cancer cells. Additionally, some changes such as reduced expression of E-cadherin, B-catenin mutations, differential MMP protein activity (i.e. more MMP2 and less MMP9 activity) and

up-regulation of SFK observed in metastatic NET-derived cells require further evaluation as therapeutic targets.

Finally, angiogenesis has been well studied in NETs, as a fundamental process in malignant transformation, tumour growth and progression. Results of many studies have indicated that VEGF expression is a prognostic factor and an interesting target for molecular therapy. At present, there are many ongoing clinical trials of angiogenesis inhibitors (including valatinib, thalidomide, pazopanib, JI-101, dovitinib, atiprimod and sorafenib) and many others are being evaluated in pre-clinical trials (for example, AF-493-NA, DC101, bevacizumab and ZK 304709). The results from these studies will be required to demonstrate that it is possible to overcome potential antiangiogenic resistance and produce long-term clinical benefit in NET patients.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-14-0106>.

Declaration of interest

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

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Author contribution statement

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