Neuroendocrine tumours: cracking the epigenetic code

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Abstract

The field of epigenetics has evolved rapidly over recent years providing insight into the tumorigenesis of many solid and haematological malignancies. Determination of epigenetic modifications in neuroendocrine tumour (NET) development is imperative if we are to improve our understanding of the biology of this heterogeneous group of tumours. Epigenetic marks such as DNA methylation at *RASSF1A* are frequent findings in NETs of all origins and may be associated with worse prognosis. MicroRNA signatures and histone modifications have been identified which can differentiate subtypes of NET and distinguish NET from adenocarcinoma in cases of diagnostic uncertainty. Historically, candidate gene-driven approaches have yielded limited insight into the epigenetics of NET. Recent progress has been facilitated by development of high-throughput tools including second-generation sequencing and arrays for analysis of the 'epigenome' of tumour and normal tissue, permitting unbiased approaches such as exome sequencing that identified mutations of chromatin-remodelling genes *ATRX/DAXX* in 44% of pancreatic NETs. Epigenetic changes are reversible and therefore represent an attractive therapeutic target; to date, clinical outcomes of epigenetic therapies in solid tumours have been disappointing; however, *in vitro* studies on NETs are promising and further clinical trials are required to determine utility of this class of novel agents. In this review, we perform a comprehensive evaluation of epigenetic changes found in NETs to date, including rare NETs such as pheochromocytoma and adrenocortical tumours. We suggest priorities for future research and discuss potential clinical applications and novel therapies.

Key Words

- ▶ Neuroendocrine
- ▶ epigenetic, methylation
- ▶ histone
- ▶ miRNA
- ▶ carcinoid
- ▶ *RASSF1*
- ▶ *ATRX/DAXX*

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Introduction

Epigenetics can be defined as the study of heritable changes in gene expression without alteration of the underlying DNA sequence. In recent years, the understanding of epigenetic drivers of tumorigenesis has developed rapidly in part due to improvements in sequencing technology. While the study of genetics – mutations, deletions and alterations to DNA – has led to many significant breakthroughs in medicine, it is increasingly apparent that for many human diseases epigenetic

drivers are key to pathogenesis and may have significant impact on clinical practice and patient management. Integration of genetics, epigenetics and gene expression can generate a holistic understanding of the molecular basis of malignancy. The recent discovery of mutations in the novel tumour suppressor genes and chromatin remodellers *ATRX/DAXX* through exome sequencing represent significant progress in our understanding of neuroendocrine tumour (NET) development.

NETs are a heterogeneous group of tumours, which until recently have remained largely intractable to genetic characterisation, with many research studies being performed with a small number of samples leading to the identification of low-frequency mutations, which do not appear to have significant prognostic or therapeutic impact. There is significance to the many negative findings in the research of NET genetics: classical tumour suppressor and oncogenes implicated in the development of many solid tumours do not appear to play a significant role in NET pathogenesis (for example *P53* (*TP53*), *RB* (*RB1*) and *KRAS* are only very infrequently mutated or deleted in NET), suggesting the presence of alternative pathogenic drivers (Yoshimoto *et al.* 1992, Yashiro *et al.* 1993, Chung *et al.* 1997a).

Here, we provide a review into the current knowledge of epigenetic changes in NET to facilitate further research and identification of potential epigenetic drivers of NET tumorigenesis and identify novel biomarkers.

Epigenetic modifications

DNA methylation is the best-understood and most stable epigenetic mark; it occurs primarily at the 5'-position of the cytosine ring within CpG dinucleotides. CpG sites are clustered in 'islands' in the promoter region of up to 70% of genes, and methylation is facilitated by DNA methyltransferase enzymes (DNMTs). Three families of DNMT have been identified: DNMT1, which maintains methylation, DNMT2, whose specific role is unknown, and DNMT3, which functions as a *de novo* methyltransferase (Bestor 1992, Okano *et al.* 1998a,b, 1999). Recent identification of conversion of 5-methylcytosine to 5-hydroxymethylcytosine by the TET group of enzymes provides further insight into the complexities of regulating gene expression, but this has not yet been investigated in NET (Tahiliani *et al.* 2009). Patterns of DNA methylation are tissue specific; therefore, comparisons of tumour and normal tissue methylation must be carefully matched to reduce artefactual results.

Histones are the primary component of chromatin and function to package DNA into nucleosomes. Post-translational covalent histone modifications regulate accessibility of genes to transcription factors by formation of binary states either 'permissive/open' or 'repressive/closed', and the establishment and maintenance of chromatin structure help to maintain cellular identity through repression of non-essential genes (Chi & Bernstein 2009). Histone modifications include acetylation, methylation and phosphorylation; these are

catalysed by several enzymes, for example histone acetyltransferases and deacetylases (HDACs).

MicroRNAs (miRNAs) are small single-stranded RNA molecules of ~22 nucleotides in length, which post-transcriptionally regulate gene expression. Over the past decade, there has been a growing interest in miRNA and their involvement in the contribution of the development of cancer, and it has been recently shown that miRNA profiling can classify human cancers (Lu *et al.* 2005). Figure 1 illustrates the primary mechanisms of epigenetic alteration. These processes work synergistically to modify gene expression and determine cellular phenotype, in particular the maintenance of the differentiated cellular state. Influence from environmental factors such as diet and smoking is known to affect epigenetic status and is likely to play a significant role in tumorigenesis (Feil & Fraga 2011).

Key epigenetic findings in cancer

Epigenetic changes are known to play a fundamental role in cancer development (Jones & Baylin 2002, Jones & Martienssen 2005). Early research identifying global hypomethylation of tumours relative to normal tissue demonstrated one of the first documented epigenetic alterations in human cancer cell lines in 1983 (Feinberg & Vogelstein 1983). Global hypomethylation increases during tumour development (Fraga *et al.* 2004) and is believed to contribute to chromosomal instability (Eden *et al.* 2003). Hypermethylation of CpG islands in the promoter regions of tumour suppressor genes has been recognised as an early event in many cancers including retinoblastoma (Greger *et al.* 1989, Sakai *et al.* 1991). Promoter hypermethylation has subsequently been identified at sites such as *VHL*, *BRCA*, *RASSF1A* and *p16INK4a* (*CDKN2A*) where associated loss of expression is seen (Herman *et al.* 1994, Merlo *et al.* 1995, Catteau *et al.* 1999, Dammann *et al.* 2000). Next-generation sequencing permits definition of full tumour 'methylomes' at single-nucleotide resolution, and there are currently large-scale international collaborative studies underway that will provide freely available epigenetic data for normal and tumour tissues; although due to their relative rarity, NETs are not included in most of these projects (The American Association for Cancer Research Human Epigenome Task Force & European Union 2008, Chadwick 2012).

Histone modifications also contribute to tumorigenesis, for example global reduction of acetylation at Lys16 (H4K16Ac) and of trimethylation at Lys20 of histone H4 (H4K20me3) is observed in several cancers (Fraga *et al.* 2005). Dimethylation of histone H3 at lysine 4

Figure 1
Mechanisms of histone modification and DNA methylation in human tissues.

(H3K4diMe) has been shown to be associated with an activated transcriptional state and is predictive of outcome in prostate and lung cancer (Seligson *et al.* 2005, Barlesi *et al.* 2007).

Epigenetic aberrations often preceded 'classical' genetic changes found in cancer such as tumour suppressor gene mutation, proto-oncogene activation and genomic instability and could therefore be utilised as early biomarkers. Hypermethylation of glutathione S transferase (*GSTP1*) is a highly specific diagnostic biomarker in prostate cancer detectable in blood and urine (Cairns *et al.* 2001) and hypermethylation of the DNA repair gene *MGMT* is a predictive biomarker of response to temozolomide (Hegi *et al.* 2005). There are few studies that have specifically investigated miRNA expression in NETs; although in other tumour types, miRNA signatures show utility as diagnostic and predictive biomarkers (Liu *et al.* 2012, Wang *et al.* 2012).

A key element driving therapeutic approaches to epigenetic modifications is the reversibility of DNA methylation and histone marks – this has been exploited successfully through histone deacetylase inhibitors (HDACi) and DNA demethylators. Two agents, 5-azacytidine and 5-aza-2'-deoxycytidine (decitabine), have been approved for treatment of myelodysplastic syndrome and leukaemia respectively (Muller *et al.* 2006,

Oki *et al.* 2007). In ovarian cancer, demethylating agents have been shown to cause resensitisation to platinum in heavily pretreated populations resulting in response rates of up to 35% and progression-free survival of 5.6–10.2 months (Fu *et al.* 2011, Matei *et al.* 2012). However, other early phase trials in solid tumours have been largely disappointing, and to date, there have been no clinical trials of these two agents in NET patients. Figure 2 provides a timeline of key developments in the field of epigenetics, alongside key NET research findings.

Pancreatic NETs

Pancreatic NETs (pNETs) comprise functioning tumours (40–55%) including gastrinoma, insulinoma, VIPoma and glucagonoma as well as non-functional NETs. Overall 5-year survival for pNET is 80% for all stages and 25% for metastatic disease; median survival is only 10 months for high-grade/poorly differentiated tumours. Improvements in prognostication and personalisation of treatment are required in order to improve patient outcomes. PNETs are perhaps the best-characterised group of NET, but despite this, reports of frequency of epigenetic marks vary significantly between studies and will require stringent validation before clinical applications can be developed. Insulinomas display more indolent clinical behaviour

Figure 2

Timeline of key developments in epigenetic research and neuroendocrine tumour research and treatment advances. Figure 2 references: ¹(Waddington 1957), ²(Friedman et al. 1963), ³(Allfrey et al. 1964), ⁴(Feinberg & Vogelstein 1983), ⁵(Greger et al. 1989, Sakai et al. 1991), ⁶(Clark et al. 1994), ⁷(Jenuwein & Allis 2001), ⁸(Lagos-Quintana et al. 2001, Calin et al. 2004), ⁹(Eckhardt et al. 2004), ¹⁰(Kaminskas et al. 2005), ¹¹(Mann et al. 2007), ¹²(Lister et al. 2008), ¹³(Bernstein et al. 2010), ¹⁴(Modlin et al. 2004), ¹⁵(Lembeck 1954, Pernow & Waldenstrom 1954), ¹⁶(Frolich et al. 1978), ¹⁷(Moertel et al. 1980), ¹⁸(Bauer et al. 1982), ¹⁹(Oberg et al. 1983), ²⁰(O'Connor & Deftos 1986), ²¹(Krenning et al. 1989), ²²(Krenning et al. 1999), ²³(Bruns et al. 2002), ²⁴(Rinke et al. 2009), ²⁵(Raymond et al. 2011, Yao et al. 2011), ²⁶(Jiao et al. 2011).

than other pNETs, and this is reflected in the emergence of distinct epigenetic patterns in this class of tumours.

DNA methylation in pNET

RASSF1A promoter region is frequently hypermethylated in pNET

The Ras-association domain gene family 1 (*RASSF1*) tumour suppressor gene acts to induce cell cycle arrest and is inactivated in a wide variety of human cancers, usually as a result of epigenetic promoter methylation, somatic mutations of the gene bring extremely rare (Shivakumar et al. 2002, Dammann et al. 2003b). Different isoforms of *RASSF1* exist and are associated with distinct promoter regions and alternative splicing. Within this review, we cite *RASSF1* as described in the original research articles.

The largest study of DNA methylation in NETs included 48 largely non-functional (94%) pNETs, which were analysed for methylation of the promoter region of 11 candidate tumour suppressor genes. The most commonly hypermethylated promoter region was that of

RASSF1A (75%), which was unmethylated in the adjacent normal tissue. Fourteen cases had synchronous hepatic metastases, the majority of which demonstrated identical methylation status as the primary tumour (House et al. 2003). Separate studies have identified *RASSF1* promoter methylation in 80–83% of pNETs making it the most frequently altered gene in sporadic pNET identified to date. In some cases, methylation is also identified in matched normal pancreas, however at a lower level of expression (Dammann et al. 2003a, Malpeli et al. 2011). *RASSF1* methylation has been noted to be more frequent in metastatic tumours (100%) than non-metastatic (71%) (Malpeli et al. 2011). Methylation of the *RASSF1* promoter occurs less frequently in pancreatic ductal adenocarcinoma (Dammann et al. 2003a).

In contrast to *RASSF1A*, the *RASSF1C* isoform expression is 11.4 times higher in pNET than in normal tissue ($P=0.001$) (Malpeli et al. 2011). *RASSF1C* has been shown to have a role inhibiting β -catenin degradation and given the above findings may also have a pathogenic role in pNET development (Estrabaud et al. 2007). *RASSF1B* is ubiquitously expressed in both tumour and normal tissue (Toyota et al. 1999).

CDKN2A promoter methylation distinguishes subtypes of pNET and may be prognostic

Cyclin-dependent kinase inhibitor 2a/*P16INK4a* is a tumour suppressor gene that plays a vital role in cell cycle regulation. Loss of heterozygosity on chromosome 9p21 (where *P16/CDKN2A* is located) is one of the most frequent genetic alterations in human cancer, and promoter methylation at this site is a common feature of many malignancies including breast (33%), prostate (60%) and colorectal cancer (92%) (Herman et al. 1995).

Methylation at the *CDKN2A/P16* locus has been demonstrated in QGP1 (a pNET cell line) (Lubomierski et al. 2001) and in 40% of pNETs (associated with early tumour recurrence and reduced overall survival) (House et al. 2003). Unlike *RASSF1*, methylation of *CDKN2A/p16INK4a* is less frequent in pNET than in adenocarcinoma (Dammann et al. 2003a). In two studies of gastrinomas, hypermethylation of the *CDKN2A/p16INK4a* promoter has been found in 52–62.5% of cases independent of disease stage, location of primary or prognosis, suggesting that methylation of the *CDKN2A/p16INK4a* promoter may be an early occurrence in gastrinoma tumorigenesis (Serrano et al. 2000, de Wilde et al. 2012). By contrast, insulinomas have been shown to have a low frequency (17%) of *CDKN2A/p16INK4a* alterations (Bartsch et al. 2000).

Methylation of other loci may be relevant to pNET development

TIMP3 promoter hypermethylation has been demonstrated in 44% of pNETs and correlated with the presence of metastases. Interestingly, none of the five insulinomas in this cohort showed methylation at this site (Wild et al. 2003). The *MGMT* promoter is methylated in 40% of pNET and may be a useful predictive biomarker of response to temozolomide chemotherapy (Dammann et al. 2003b). Only 6% of pNETs show hypermethylation of *VHL* (compared with 18% showing deletion of the gene), suggesting that *VHL* promoter methylation is not a significant mechanism of tumorigenesis (Schmitt et al. 2009).

CpG island methylator phenotype positivity is a poor prognostic marker in pNET

CpG island methylator phenotype (CIMP) positivity (a term denoting a high degree of methylation across multiple CpG sites) in 20% of a cohort of 71 mixed

pancreatic and gastrointestinal NETs corresponded with higher grade tumours (Ki67 > 10%), while CIMP negativity was associated with improved overall survival (Arnold et al. 2007). This is in keeping with methylation at three or more loci of selected candidate genes being associated with early recurrence and worse prognosis in a separate study (House et al. 2003).

Insulinomas are distinguished from other pNET subtypes through differences in DNA methylation

A large study with a mixed cohort of 62 NET cases were analysed for DNA methylation at three regions associated with the *IGF2* gene previously identified as differentially methylated in mouse models (Fontaniere et al. 2006, Dejeux et al. 2009). The gastrinomas, non-functional pNETs and small intestinal NETs in the cohort exhibited hypomethylation of varying degrees at these sites; by contrast, the 11 insulinomas were hypermethylated at these regions. This correlated with insulinoma-specific overexpression of *IGF2* RNA and protein thought to be caused by loss of imprinting and reactivation of the normally silent maternal allele (Dejeux et al. 2009).

Promoter methylation of *MLH1* has been identified in 31% of insulinomas and correlated with poor prognosis (Mei et al. 2009), and unlike most pNETs, *TIMP3* and *CDKN2A/p16INK4a* methylation are not common features of insulinomas (Bartsch et al. 2000, Wild et al. 2003). In Table 1, we summarise genes known to be methylated in pNET as well as other anatomical origins.

Chromatin remodellers in pNET

In a recent study (Jiao et al. 2011), exome sequencing was performed in ten sporadic pNETs followed by Sanger sequencing validation in a cohort of 58 pNETs identifying commonly mutated genes implicated in chromatin remodelling. Forty-four per cent of tumours had inactivating mutations of *MEN1* and 43% had mutations in either *DAXX* or *ATRX* (25 and 18% respectively) (death domain-associated protein or alpha thalassemia/mental retardation syndrome X-linked) (Jiao et al. 2011).

MEN1 encodes the transcription factor menin, which recruits the H3K4me3 histone methyltransferase mixed lineage leukaemia complex that plays an essential role in chromatin remodelling (Agarwal et al. 1999). *ATRX* and *DAXX* had not previously been associated with NETs and are novel tumour suppressor genes, although *ATRX* has previously been implicated in glioma. The proteins encoded by *ATRX* and *DAXX* have multiple functions

Table 1 Summary of loci identified with aberrant methylation in neuroendocrine tumours.

Locus	Association			Reference
	Pancreatic NET	GI NET	Bronchial NET	
<i>CDKN2a/p16INK4a</i>	HPM 62.5% gastrinomas HPM 50% non-functional HPM 52% gastrinomas HPM 11.8% insulinomas HPM in QGP1 but not BON HPM 17% HPM 0% insulinomas	HPM 0% appendiceal HPM 84.8% metastatic HPM 60.6% non-metastatic	HPM NCI-H727 and HTB-119	Muscarella et al. (1998) Muscarella et al. (1998) Serrano et al. (2000) Bartsch et al. (2000) Lubomierski et al. (2001) Dammann et al. (2003a) Dammann et al. (2003a) Zhang et al. (2006)
<i>RASSF1</i>	HPM 83% HPM 75% HPM 63% HPM 32% (foregut GEP) HPM 71.3% (foregut GEP) HPM 71% fore-midgut GEP, 38% colorectal HPM 80%	HPM 69% HPM 20–40% colorectal	HPM 40% HPM promoter 1	Zhang et al. (2006) Zhang et al. (2006) Dammann et al. (2003a) House et al. (2003) Liu et al. (2005) Pizzi et al. (2005) Arnold et al. (2007) Arnold et al. (2008) Pelosi et al. (2010) Malpeli et al. (2011) La Rosa et al. (2012)
<i>TIMP3</i>	HPM 0% HPM 44% HPM 0% insulinomas HPM 0% (foregut GEP) HPM 0% fore-midgut GEP, 9% colorectal	HPM 20–40% colorectal HPM 25%		House et al. (2003) Wild et al. (2003) Wild et al. (2003) Arnold et al. (2007) Arnold et al. (2008) La Rosa et al. (2012)
<i>MGMT</i>	HPM 40% HPM 13% HPM 16.1% (foregut GEP) HPM 21% fore-midgut GEP, 38% colorectal	HPM 0%	HPM 27%	Chan et al. (2003) House et al. (2003) Liu et al. (2005) Arnold et al. (2007) Arnold et al. (2008)
<i>hMLH1</i>	HPM 23% HPM 0% (foregut GEP) HPM 0% fore-midgut GEP, 12% colorectal HPM 31% insulinomas	HPM 25%	HPM 33%	Liu et al. (2005) House et al. (2003) Arnold et al. (2007) Arnold et al. (2008) Mei et al. (2009)
<i>P16</i>	HPM 19% HPM 40% HPM 0% (foregut GEP) HPM 0% fore-midgut GEP, 24% colorectal			Arnold et al. (2008) House et al. (2003) Arnold et al. (2007) Arnold et al. (2008)
<i>APC</i>	HPM 21% HPM 54.1% (foregut GEP) HPM 63% fore-midgut GEP, 44% colorectal	HPM 20–40% colorectal HPM 0% appendiceal HPM 57.6% metastatic HPM 27.3% non-metastatic		La Rosa et al. (2012) Zhang et al. (2006) Zhang et al. (2006) Zhang et al. (2006)
<i>CTNNB1</i>				Arnold et al. (2007) Arnold et al. (2008) La Rosa et al. (2012)
<i>HIC1</i>	83.1% (foregut GEP) HPM 55% fore-midgut GEP, 47% colorectal	HPM 20–40% colorectal		House et al. (2003) Arnold et al. (2007)
<i>E-cadherin</i>	HPM 23% HPM 1.3% (foregut GEP)			Chan et al. (2003) La Rosa et al. (2012)
<i>RARβ</i>		HPM 25% HPM 20–40% colorectal		Arnold et al. (2007)
<i>MEN1</i>	HPM 14.3% (foregut GEP) HPM 18% fore-midgut GEP, 44% colorectal			Chan et al. (2003) La Rosa et al. (2012) Arnold et al. (2007)
<i>PTEN</i>	HPM 1.5% (foregut GEP)	HPM 20–40% colorectal		Arnold et al. (2007) La Rosa et al. (2012)

Table 1 Continued

Locus	Association			Reference
	Pancreatic NET	GI NET	Bronchial NET	
P14	HPM 0%	HPM 69%	HPM 33%	House et al. (2003)
GATA5	HPM 44%	HPM >80% colorectal		Liu et al. (2005)
ESR1		HPM 80% colorectal		La Rosa et al. (2012)
GST	HPM 0%			La Rosa et al. (2012)
RUNX3	HPM 6.6% (foregut GEP)			House et al. (2003)
P14		HPM 44%		Arnold et al. (2007)
THBS1		HPM 44%		Chan et al. (2003)
RAR (RARA)	HPM 25%			Chan et al. (2003)
P73 (TP73)	HPM 17%			House et al. (2003)
VHL	HPM 6%			House et al. (2003)
WT1		HPM 60–80% colorectal		Schmitt et al. (2009)
CDH13		HPM 40–60% colorectal		La Rosa et al. (2012)
CIMP status	CIMP+ve 29% fore-midgut GEP, 59% colorectal			La Rosa et al. (2012)
				Arnold et al. (2008)

HPM, hypermethylated; NET, neuroendocrine tumour; GEP, gastroenteropancreatic; MSP, methylation-specific PCR; GI, gastrointestinal; DMR, differentially methylated region; DNMT, DNA methyltransferase; CIMP, CpG island methylator phenotype; MANEC, mixed adenoneuroendocrine carcinoma.

including chromatin remodelling during heterochromatin assembly at repetitive guanine-rich regions (such as telomeres) where they are required for incorporation of the histone variant H3.3 (Heaphy et al. 2011a). Mutations in *ATRX* and *DAXX* were mutually exclusive but overlapped with *MEN1* mutations in 16 cases (23.5%). The presence of mutations in *ATRX/DAXX* was associated with improved prognosis.

NETs harbouring *ATRX/DAXX* mutations have been shown to demonstrate alternative lengthening of telomeres (ALT), indicating that they do not increase activity of telomerase in the face of extensive cell proliferation but maintain telomeres through alternative mechanisms (Heaphy et al. 2011a). All 19 tumours with *ATRX/DAXX* mutations from the Jiao study were found to have ALT positivity – a 100% correlation ($P < 0.008$ for each gene), while in human cancers in general, the ALT phenotype is present in only 3% (Heaphy et al. 2011b). The association of *ATRX/DAXX* with ALT has subsequently been confirmed in other tumour types including glioblastoma and osteosarcoma substantiating the role of *ATRX/DAXX* in telomere maintenance (Schwartzentruber et al. 2012). ALT phenotype has been found to be a favourable prognostic factor, possibly due to the reduction of chromosomal instability observed in these tumours (Ulaner et al. 2003).

The role of *ATRX/DAXX* was subsequently investigated in 28 multiple endocrine neoplasia 1 (*MEN1*) syndrome-related pNETs, loss of *ATRX/DAXX* expression occurred in only 6% of these tumours, all of which

were >3 cm in size and demonstrated ALT phenotype, suggesting that *ATRX/DAXX* mutation is a late event in *MEN1*-associated pNET development (de Wilde et al. 2012). Forty-seven microadenomas (<0.5 cm) were included in the study, none of which showed *ATRX/DAXX* mutation or ALT positivity.

There has been little research investigating histone modifications in pNETs; however, given the frequency and prognostic impact of modifications related to chromatin remodelling, it is recommended that histone modifications should be studied more extensively in pNETs. One study was identified assessing mutation or aberrant expression of HDAC11 (selected for study due to its position at locus 3p25 which is associated with a high rate of loss of heterozygosity in pNETs) in a small cohort of sporadic malignant pNETs detected no tumour specific events suggesting that HDAC11 is unlikely to play a role in these tumours (Chung et al. 1997b, Barghorn et al. 2001).

miRNA expression in pNET

miRNAs 103/107/155 can distinguish pNET from normal pancreatic tissue

Roldo et al. (2006) investigated the global expression of miRNAs in pNETs. Analysis of global miRNA expression of 44 pancreatic primary tumours determined a common pattern of miRNA expression distinguishing pNETs from normal pancreas: expression of miR-103 and miR-107 associated with lack of expression of miR-155.

Overexpression of miR-204 was specific to insulinomas and correlated with immunohistochemical expression of insulin. MiR-21 was strongly associated with a high Ki67 proliferation index and liver metastases and may be a useful prognostic marker.

Gastrointestinal NETs

Approximately two thirds of gastroenteropancreatic NET arise from the intestine, these comprise foregut, the classical 'midgut' NETs, and the typically higher grade hindgut and rectal NETs, which are associated with poorer prognosis. Interest in the methylation status of GI NET has been encouraged by understanding of the role of methylation and CIMP in colorectal adenocarcinoma. Methylation of certain loci in GI NET appears to be related to metastatic progression, and there may be a distinct subgroup of colorectal cancer patients with CIMP+/MSI+ who experience an improved overall prognosis. The limited studies of histone modifications in these tumours have been less informative.

DNA methylation in gastrointestinal NET

Gastrointestinal and pNETs have different methylation patterns

One of the earliest studies of DNA methylation in NET compared with CpG island methylation in 16 GI NETs and 11 pNETs showed differing profiles of methylation across a panel of 14 candidate genes commonly implicated in gastrointestinal malignancy, reflecting differing molecular pathogenesis. Specifically, GI NETs were found to be significantly more frequently methylated at the promoter regions of the following genes compared to pNETs: *MGMT* (25 vs 0% $P=0.03$), *THSB1* (44 vs 9% $P=0.04$), *P14* (44 vs 9% $P=0.04$), *P16* (31 vs 9%) and *RAR β* (25 vs 0% $P=0.03$). pNETs were more frequently methylated at *ER* (64%), and of note, *MEN1* was not methylated in any of the samples (Chan *et al.* 2003).

Global hypomethylation is a feature of gastrointestinal NET

Methylation of repeat elements such as long interspersed nucleotide elements (*LINE1* (*L1RE1*)) and *Alu* is a surrogate for global methylation levels. A study of 35 NETs (15 ileal, 11 pancreatic and nine others) demonstrated that all tumour samples were hypomethylated relative to normal tissue at these sites (*LINE1* $P=0.04$, *Alu* $P=0.001$).

Global hypomethylation was more prevalent in GI NET than pancreatic and was correlated with poor prognosis, lymph node metastasis and loss of chromosome 18 (Choi *et al.* 2007).

However, in a more recent study of 43 GI NETs and 15 pNETs, *LINE1* hypomethylation was identified in 100% pNETs, 50% gastric NETs, 82% midgut NETs and 100% colorectal NETs while NETs metastatic to lymph nodes were less frequently hypomethylated than non-metastatic NET (Stricker *et al.* 2012). We can conclude that global hypomethylation is a feature of pNETs, and while it is generally considered a poor prognostic marker in most solid tumours, the prognostic implication in pNET remains to be determined.

RASSF1A and *CTNNB1* promoters are frequently methylated in gastrointestinal NETs

Two interesting studies of *RASSF1A* methylation in GI NET have been performed. In the first study, *RASSF1A* methylation was identified in 32% (20/62) GI NETs and was restricted to foregut tumours (Pizzi *et al.* 2005). The second study determined that frequency of *RASSF1A* methylation increased in metastatic lesions compared with primary tumours in a study of 33 midgut NET cases (61% methylated in primary tumours vs 85% in matched metastatic lesions). *CTNNB1* promoter methylation is also more frequent in metastatic than in primary GI NET tumours (57.6 vs 27.3% $P=0.004$) and is not seen in normal tissue (Zhang *et al.* 2006). None of the six appendiceal NETs included in the study (all non-metastatic as most appendiceal NETs) displayed *RASSF1A* or *CTNNB1* methylation (Zhang *et al.* 2006). These results suggest that *RASSF1A* may be involved in the development of endocrine tumours of the foregut and midgut but not hindgut and that *RASSF1A/CTNNB1* may be associated with progression to metastasis.

CIMP may be a prognostic biomarker for colorectal NET

Recent studies have demonstrated CIMP positivity in 37–59% of poorly differentiated colorectal NETs (Arnold *et al.* 2008, La Rosa *et al.* 2012) compared with 29% of well-differentiated fore/midgut NETs. In one study, survival was not influenced by CIMP status; however, La Rosa *et al.* demonstrated a 100% correlation of CIMP positivity with the presence of microsatellite instability, and this was associated with better prognosis (MSI/CIMP+ve vs others $P=0.04$).

Expression of DNMT1, -3A and -3B has been demonstrated to be significantly higher in stage IV GEP NET than in stage I or II. DNMT3A (a *de novo* methyltransferase) was significantly higher in poorly differentiated carcinomas relative to well-differentiated tumours ($P < 0.01$), and DNMT3A and -3B both showed significantly lower expression in midgut NET relative to fore- or hindgut tumours. We can conclude that overexpression of DNMT1, -3A and -3B is a common feature of GEP NET, and these enzymes may play a role in the development of NET and are therefore a potential novel therapeutic target (Rahman et al. 2010). The relationship between DNMT activity and CIMP is not fully characterised and further large studies are required to resolve the relationship between the activity of these enzymes, methylation status and prognosis in GI NET.

Methylation of other loci may be relevant in colorectal NET

In a study including 34 colorectal and 38 fore-midgut NETs, promoter methylation of *CDKN2A/p16INK4a*, *hMLH1* and *TIMP3* was only detected in NET of colorectal origin. *CDKN2A/p16INK4a* methylation was only present in poorly differentiated colorectal NET ($P = 0.01$) and was a more significant prognostic marker of poor outcome than Ki67 ($P = 0.0004$) (Arnold et al. 2008). Altered expression of *MLH1* by both genetic and epigenetic mechanisms is an area of exciting research in colorectal adenocarcinoma and may be significant in the development of both endocrine and exocrine tumours of this site. Methylation of the *GATA4/5* promoter (a gene known to be frequently methylated in colorectal adenocarcinoma) is present in >90% of colorectal NET and MANEC (La Rosa et al. 2012). Table 1 provides a summary of loci differentially methylated in GI NET.

Histone modifications in gastrointestinal NET

Research of histone modifications in NETs is limited to studies on small numbers of tumours. H3K4diMe and the associated methylating (Ash2 complex) and demethylating (LSD1) enzymes were studied in gastrointestinal carcinomas including a group of 16 mostly low-grade primary intestinal NETs (Magerl et al. 2010). Up to 100% of neuroendocrine carcinomas exhibited strong immunostaining for Ash2 and LSD1, while H3K4diMe was strongly expressed in 93% of neuroendocrine carcinomas, other carcinomas (such as HCC and pancreatic adenocarcinomas) showing lower expression. In matched normal tissue, all three markers showed weak expression. This suggests a

potential role for modifications at the histone H3K4 locus in intestinal NETs but requires further validation.

Histone H1x has been demonstrated to be highly expressed in NETs but also in the corresponding non-neoplastic neuroendocrine cells of the pancreas and small intestine and therefore may reflect cell of origin rather than tumorigenic feature (Warneboldt et al. 2008).

miRNA expression in gastrointestinal NET

MiR-133a is down-regulated in metastatic progression of ileal NET

Two miRNA expression profile analyses provide evidence that miRNAs are dysregulated specifically in ileal NET progression: down-regulation of miR-133a, -145, -146, -222 and -10b and up-regulation of miR-183, -488 and -19a + b in metastatic tumours compared to primary tumours (Ruebel et al. 2010) and up-regulation of miR-96, -182, -183, -196a and -200a and down-regulation of miR-31, -129-5p, -133a and -215 (Li et al. 2012). These results suggest that miRNAs may play a role in GI NET progression, in particular miR-133a that was identified in both studies and may be a useful focus for further investigation.

miRNAs can distinguish colorectal NET from adenocarcinoma

A case of colorectal NET was included in comparison of miRNA expression in colorectal adenocarcinoma and normal mucosa, which identified 38 up-regulated miRNAs in colorectal NET compared with the normal mucosa. Of these, only six were found to be up-regulated in colorectal adenocarcinoma, suggesting distinct miRNA expression pattern in colorectal NET compared with adenocarcinomas (Hamfjord et al. 2012). Four miRNAs identified as up-regulated in colorectal NET were also noted in the analyses of ileal NET described earlier: miR-96, -182, -196a and -488; these would be good candidates for further investigation; however, it must be noted that this study only analysed one NET case and analysis of a larger sample size and validation of the data is required.

Bronchial NETs

Bronchial NETs can be classified by cellular morphology into small-cell (SCLC), large-cell (LCNEC), 'atypical' carcinoid (AC) and 'typical' carcinoid (TC) tumours. In this review, we focus on the typical and atypical carcinoid subtypes of bronchial NET.

DNA methylation in bronchial NET

The *RASSF1* promoter is hypermethylated in bronchial NET

Analysis of methylation of the *RASSF1* promoter regions in a cohort of 58 bronchial NETs, matched normal tissue and 20 control non-small-cell lung cancers (NSCLC) identified that promoter 1 was hypermethylated in NET but not normal tissue or non-endocrine tumours. The degree of hypermethylation correlated with tumour grade and corresponding global loss of *RASSF1A/E* mRNA expression (Pelosi *et al.* 2010), and *RASSF1* promoter 1 methylation may represent a useful biomarker in lung NET to differentiate high- from low-grade NET. Up-regulation of *RASSF1C* was found to be an independent adverse prognostic factor in high-grade bronchial NETs (Pelosi *et al.* 2010).

P15.5 promoter methylation may be implicated in low-grade bronchial NET

Similar to the tumour suppressor gene *CDKN2A/p16INK4a*, *P15INK4b* (*CDKN2B*) also encodes a cyclin-dependent kinase inhibitor, which functions in cell cycle regulation, but its role in tumorigenesis is less well characterised. A series of five low-grade and 15 high-grade bronchial NETs showed aberrant methylation at the 5'-region of the *p15INK4b* gene in 15% of tumours but not in control/normal lung tissue, and there was a highly variable expression of isoform *p15.5* in the low-grade bronchial NETs, suggesting potential involvement of this gene in the 'carcinoid'-type bronchial tumours. *P15* status was shown to be independent of *P16* and *P14* (Chaussade *et al.* 2001).

Histone modifications in bronchial NET

Histone 4 modifications can differentiate low- and high-grade bronchial NETs

A case series of 32 bronchial NETs demonstrated progressive loss of two different histone marks, H4KA16 and H4KM20, from low- to high-grade tumours, and found that Ki67 was inversely correlated with both H4KA16 and H4KM20 ($P < 0.05$) (Li *et al.* 2011).

EZH2 is strongly expressed in high-grade bronchial NET

Increased expression of enhancer of zeste homolog 2 (*EZH2*), a molecule involved in methylation of histone H3 (H3K27), has been demonstrated in many human malignancies and is associated with poor prognosis

(Bachmann *et al.* 2006). *EZH2* was demonstrated to be strongly expressed in all SCLC and LCNEC (80–90% of cells) in a cohort of 54 bronchial NETs, but only rare scattered expression was seen in typical and atypical bronchial NETs (Findeis-Hosey *et al.* 2011). *EZH2* may play a role in the development of high-grade NETs and may be a useful diagnostic biomarker.

miRNA expression in bronchial NET

miRNA-21 and -155 distinguish high- and low-grade bronchial NETs

In a recent study evaluating expression of selected miRNAs in 63 bronchial NETs, miRNA-21 and -155 were found to be up-regulated in high-grade tumours but not in TC/AC, and miRNA-21 was found to be significantly up-regulated in metastatic low-grade NETs compared with non-metastatic tumours (Lee *et al.* 2012). These results require validation but suggest that miRNA profiles may be utilised as diagnostic and prognostic biomarkers.

Table 2 summarises the current state of knowledge of the role of miRNA in NET. Interestingly, miRNA-155 has been identified as a biomarker of both pancreatic (down-regulated) and bronchial NETs (up-regulated), tumours of foregut embryological origin. A definitive role for miRNA involvement in NET development and signalling pathways still needs to be determined and further validation studies were performed with larger sample sets.

Rarer NETs

There is limited research into the impact of epigenetic changes on rare NET subtypes, and there are no published reports of any epigenetic research performed on thymic NETs.

Hypermethylation of *RASSF1A* and *p14ARF* promoter regions is frequent in Merkel cell carcinomas

The pathogenesis of Merkel cell carcinomas (MCCs) is poorly understood. Two studies have been performed investigating epigenetics in MCC, both candidate-driven analyses of promoter methylation, which demonstrated promoter hypermethylation of *RASSF1A* (51%), *p14ARF* (42%) and *CDKN2A/p16INK4a* (5–22%) (Lassacher *et al.* 2008, Helmbold *et al.* 2009). The Merkel cell polyomavirus has recently been demonstrated to encode and express an miRNA MCV-miR-M1-5p in 50% of MCCs, which may play a pathogenic role in tumour development (Lee *et al.* 2011). No correlation was demonstrated

Table 2 Summary of miRNA associated with neuroendocrine tumours.

miRNA	Association			Reference
	Pancreatic NET	GI NET	Bronchial NET	
miR-204: up-regulated	Associated with insulinoma Distinguishes pNET from normal			Roldo et al. (2006)
miR-103, -107: up-regulated				Roldo et al. (2006)
miR-155: down-regulated miR-10b, -133a, -145, -146, -122: down-regulated miR-183, -488, -19a + b: up-regulated miR-31, -129-5p, -133a, -125: down-regulated		Associated with metastatic ileal NET		Ruebel et al. (2010)
miR-96, -182, -183, -196a, 200a: up-regulated miR-653, -7, -489, 1224-5p: up-regulated		Associated with metastatic small intestinal NET		Li et al. (2012)
miR-21, -155: up-regulated		Associated with colorectal NET (vs normal mucosa)	Associated with high-grade NET	Hamfjord et al. (2012) Lee et al. (2012)

NET, neuroendocrine tumour; miRNA, microRNA.

between polyomavirus infection and methylation status (Helmbold et al. 2009).

Promoter hypermethylation of *CDKN2A/p16INK4a* is prognostic in paragangliomas

Promoter hypermethylation of *CDKN2A/p16INK4a* is present in 100% of paragangliomas with succinate dehydrogenase complex subunit B (*SDHB*) mutation and is associated with metastasis and poorer prognosis. MEN2/RET-associated paragangliomas in contrast did not exhibit significant hypermethylation (Kiss et al. 2008, 2013). CIMP positivity is present in 9% of paragangliomas and is associated with malignant behaviour (Geli et al. 2008).

Global hypomethylation and *IGF2* promoter hypermethylation are associated with malignant behaviour of adrenocortical tumours

Malignant adrenocortical tumours (ACTs) are globally hypomethylated relative to benign tumours (which shared most of their methylation patterns with normal tissue). Two groups have analysed hypermethylation in ACT identifying 212 hypermethylated CpG islands and 52 hypermethylated promoter loci respectively. Genome wide profiling of ACT can distinguish methylation patterns of normal and malignant tumours, specifically hypermethylation of *CDKN2A* and *GATA4* is present in

ACT but not in normal tissue, while *IGF2* promoter hypermethylation is identified in malignant ACT but not in normal tissue or benign tumours (Fonseca et al. 2012, Rechache et al. 2012). CIMP positivity in ACT has been associated with poor survival (Barreau et al. 2013). Treatment of an ACT cell line (NCI-H295R) with decitabine restored expression of hypermethylated genes and has anti-proliferative effects (Suh et al. 2010, Fonseca et al. 2012).

miRNA profiling distinguishes malignant and benign ACTs

Several investigations of the role of miRNA in ACTs have been performed in recent years and have demonstrated association of malignant ACT with the following expression patterns: down-regulation of miR-100, -125b and -195 and up-regulation of miR-483-5p (Patterson et al. 2011); up-regulation of miR-335 and -675 (Schmitz et al. 2011); up-regulation of miR-195 and -483-5p (Soon et al. 2009); up-regulation of miR-184 and -503 and down-regulation of miR-511 and -214 (Tombol et al. 2009); up-regulation of miR-503, -1202 and -1275 (Ozata et al. 2011). Inhibition of miR-483-3p or -483-5p and over-expression of miR-195 or -497 reduced cell proliferation in human NCI-H295R ACT cells (Ozata et al. 2011). A comprehensive review of dysregulation of miRNA in ACTs has recently been published (Singh et al. 2012).

11p15.5 imprinted genes may be pathogenic in pheochromocytoma

Loss of imprinting of the 11p15.5 allele (which contains *IGF2*) has been identified in epigenetic analysis of pheochromocytomas (both sporadic and VHL associated) (Astuti et al. 2005, Margetts et al. 2005). Other promoter regions found to be aberrantly methylated in pheochromocytoma include *HIC1* (82%) and *CASP8* (31%) (Margetts et al. 2005); *HSP47* (*SERPINH1*) (52%), *HOXA9* (17%) and *OPCML* (12%) (Margetts et al. 2008). Methylation patterns were found to be similar in neuroblastoma tumours (Margetts et al. 2005). Promoter regions of *VHL*, *SDHB* and *SDHD* are demonstrably unmethylated in sporadic pheochromocytomas (Cascon et al. 2004).

miRNA profiling can identify malignant and recurring pheochromocytomas

Three comparative analyses of benign and malignant pheochromocytoma miRNA profiles have been performed in recent years. Overexpression of miR-483-5p, -183 and -101 (Patterson et al. 2012); overexpression of miR-483-5p and underexpression of miR-15a and -16 (Meyer-Rochow et al. 2010); and overexpression of miR-1225-3p (Tombol et al. 2010) have been identified as markers of malignant or recurring tumours. MiR-483-5p has been identified in investigation of both pheochromocytomas and ACTs and is an interesting candidate for further investigation given its location at 11p15.5 within the second intron of *IGF2* and implication in other solid malignant tumours (Meyer-Rochow et al. 2010).

Therapeutic approaches using epigenetic targets

The use of novel targeted epigenetic therapies is an attractive concept due to the prevalence of modifications found in malignant tumours, and the inherent reversibility of DNA methylation and chromatin modifications. Despite this, epigenetic therapies have so far shown limited efficacy in solid tumours; however, preclinical and early clinical trials in NET are promising.

DNMT inhibitors suppress NET cell growth

The DNMT inhibitor azacytidine has been shown to cause a dose-dependent reduction in tumour cell proliferation in three NET cell lines (midgut: CDNT2.5, bronchial: NCI-H727 and pancreatic lymph node metastasis: BON1). Growth inhibition was associated by significant

reduction in chromogranin A and neuron-specific enolase (Alexander et al. 2010). Two bronchial NET cell lines (NCI-H727 bronchial 'carcinoid' and HTB-119 SCLC) showed re-expression of *RASSF1A* mRNA after treatment with decitabine (Zhang et al. 2006), while treatment of QGP1 pNET cell lines with decitabine restored *CDKN2A/p16INK4a* and caused significant growth inhibition (Habbe et al. 2007). The anti-proliferative effect is likely to be due to restoration of multiple genes silenced by pathogenic *de novo* methylation. Differential expression of 48 genes has been identified in QGP1 cells after treatment with decitabine, including 23 in which expression decreased after treatment. Many of these genes are known to be involved in cellular proliferation, apoptosis and development of metastasis and may play a role in pNET development (Habbe et al. 2007).

HDACis suppress NET cell growth

Functional analysis of three different HDAC inhibitors – trichostatin A, sodium butyrate and entinostat – has been carried out on neuroendocrine cell lines (insulinoma: CM and pancreatic lymph node metastasis: BON1) showing dose-dependent inhibition of proliferation, cell cycle arrest and induction of apoptosis. The addition of somatostatin or octreotide did not affect the outcome (Baradari et al. 2006).

Valproic acid (VPA) (a class I and IIa HDAC inhibitor) in combination with lithium chloride has been shown to suppress chromogranin A levels while reducing cellular growth in pancreatic (BON) and pulmonary (NCI-H727) NET cell lines (Adler et al. 2009), while VPA alone up-regulates notch1 and suppresses growth in gastrointestinal and bronchial NET cell lines (Greenblatt et al. 2007). Notch1 is a transmembrane receptor that translocates to the nucleus upon ligand binding and regulates gene transcription, acting as a tumour suppressor in some human cancers (Radtke & Raj 2003). Activation of Notch1 has been shown to suppress growth in NET cell lines and is associated with reduction of NET tumour markers (Kunnimalaiyaan et al. 2006).

Early phase clinical trials of HDACis in NET are inconclusive

A pilot phase II trial of VPA in low-grade NET was conducted in eight patients (two pNETs and six midgut NETs) receiving 500 mg oral VPA twice per day with dose adjustment to maintain therapeutic serum levels. Notch1 signalling was absent in all tumours before treatment and was up-regulated with VPA. One patient had an unconfirmed partial response and four had stable disease, tumour markers improved in five out of seven (Mohammed et al. 2011). This well-tolerated agent may have a role in the

management of low-grade NETs and is currently being trialled in many different tumour types.

Two trials of HDAC inhibitors have been terminated early: in 2006, depsipeptide was associated with an unexpectedly high rate of cardiac adverse events and a phase II trial in NET patients was terminated (Shah *et al.* 2006); and in 2012, a phase II trial panobinostat in a mixed cohort of 15 GEP NET patients was terminated due to lack of apparent benefit. In this single-arm trial, all patients received panobinostat 20 mg once daily three times per week. The stable disease rate was 92.3% and median progression-free survival was 11.8 months; however, response rate was 0% and stabilisation rate needs to be considered in context of the typical slow growth of low-grade NET. Thrombocytopaenia and fatigue were the most frequent toxicities (Rajguru *et al.* 2012).

While *in vitro* results of epigenetic therapies on NET cell lines appear promising, clinical trials have so far not demonstrated significant benefit to patients but are limited by the rarity of NET and challenges of performing early phase trials in this patient group. Demethylating agents have not yet been trialled in the NET population and the field of miRNA therapeutics is still in its infancy. Predictive biomarkers are required to assist patient stratification for this novel class of therapeutics, and as specificity of the agents to target a particular epigenetic change improves so, toxicity is likely to reduce and patient benefit increase.

Summary

Epigenetic changes are likely to play a key role in NET development and progress is being made in identifying potential diagnostic and prognostic epigenetic biomarkers for these tumours. The apparent disparity in epigenetic states of NETs of different cellular origins is intriguing and is likely to reflect diverse tumorigenic processes. Increased promoter methylation of *CDKN2A/P16INK4a* is a typical feature of gastrinomas, while methylation of *IGF2* and *MLH1* appears to be characteristic of insulinoma. This has implications for targeted therapeutics and personalisation of patient management and needs to be further explored in large-scale studies. Methylation of *RASSF1A* is a frequent finding in NETs of all origins and has prognostic impact, suggesting a potential 'driver' role in development of these tumours and a potentially effective drug target. CIMP positivity is associated with worse prognosis in pNETs but improved prognosis in colorectal NET. *EZH2* expression distinguishes between high- and low-grade bronchial NETs, as do histone modifications H4KA16 and K4KM20.

Characteristic miRNA signatures of NET of all subtypes are being identified that distinguish tumour from normal tissue, as well as some with prognostic significance.

A key development in our understanding of pNET has been the identification of *ATRX/DAXX* mutations in 43% of tumours and the associated ALT phenotype (Jiao *et al.* 2011). These are associated with improved prognosis in pNET and are suitable for clinical application as a prognostic biomarker. The identification of these mutations and the subsequent chromatin remodelling illustrate the synergistic nature of genetic and epigenetic regulation.

The field of epigenetic-targeted agents is still in development, and despite promising *in vitro* results in many solid tumours the only real clinical applications with FDA approval so far have come in haematological malignancies. However, there is enormous interest and investment, and with many novel agents entering clinical trials, and increasing personalisation/targeting of therapy, we anticipate that positive results in solid tumours will soon follow.

We have presented a comprehensive overview of current understanding of epigenetic changes in NETs. In many cases, the data presented is identified through small candidate-driven studies of mixed cohorts of NET cases; in consequence, some outcomes appear conflicting and drawing decisive conclusions is difficult. There are multiple mechanisms regulating gene transcription and cellular proliferation in NET and identifying the most powerful tumorigenic drivers within the group is challenging. Commonly mutated oncogenes in other solid tumours play little or no pathogenic role in NET and therefore epigenetic changes are good candidates for pathogenic drivers. The findings presented in this review give insight into the pathobiology of NET and provide rationale for the application of novel therapeutics and further avenues of research. There is clear requirement for further collaborative large-scale genome wide integrated (epi)genetic studies, the results of which will clarify what is currently a complex field of research.

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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