Genetic and epigenetic drivers of neuroendocrine tumours (NET)

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Abstract

Neuroendocrine tumours (NET) of the gastrointestinal tract and the lung are a rare and heterogeneous group of tumours. The molecular characterization and the clinical classification of these tumours have been evolving slowly and show differences according to organs of origin. Novel technologies such as next-generation sequencing revealed new molecular aspects of NET over the last years. Notably, whole-exome/genome sequencing (WES/WGS) approaches underlined the very low mutation rate of well-differentiated NET of all organs compared to other malignancies, while the engagement of epigenetic changes in driving NET evolution is emerging. Indeed, mutations in genes encoding for proteins directly involved in chromatin remodelling, such as DAXX and ATRX are a frequent event in NET. Epigenetic changes are reversible and targetable; therefore, an attractive target for treatment. The discovery of the mechanisms underlying the epigenetic changes and the implication on gene and miRNA expression in the different subgroups of NET may represent a crucial change in the diagnosis of this disease, reveal new therapy targets and identify predictive markers. Molecular profiles derived from omics data including DNA mutation, methylation, gene and miRNA expression have already shown promising results in distinguishing clinically and molecularly different subtypes of NET. In this review, we recapitulate the major genetic and epigenetic characteristics of pancreatic, lung and small intestinal NET and the affected pathways. We also discuss potential epigenetic mechanisms leading to NET development.

Introduction

Neuroendocrine tumours (NET) are a rare and heterogeneous group of tumours with widely varying morphologies and behaviours. NET originate from neuroendocrine cells throughout the body. They include endocrine tumours of the thymus, pituitary, adrenal gland, lung, pancreas, gastrointestinal tract, sympathetic and parasympathetic paraganglia, C-cells of the thyroid and uncommon localizations such as ovaries and ear. The phenotypic similarity with neurons is demonstrated by neuroendocrine markers, such as chromogranin A and synaptophysin, which are used in the histopathological diagnosis. NET are classified as functional or non-functional, depending on syndromes due to inadequate hormone secretion. While functional NET can induce dramatic clinical symptoms due to the overproduction of endogenous hormones or vasoactive substances, non-functional NET are endocrinologically silent and therefore often locally advanced or metastasized at the time of detection. The annual incidence of neuroendocrine tumours is estimated to be 3.65 per 100,000 people
(Yao et al. 2008, Lawrence et al. 2011), but due to better diagnostic tool and increased lifespan, the incidence and prevalence of these tumours is increasing.

Classification of gastroenteropancreatic (GEP) and pulmonary NET has historically been challenging and steadily evolving. In 2010, the WHO classification incorporated the existence of clinicopathological differences among NET according to the site of origin and the notion that NET should be always considered as potentially malignant (Bosman et al. 2010). The upcoming WHO 2017 classification for pancreatic neuroendocrine neoplasms (NEN) will further underline the biological difference between well-differentiated NET and poorly differentiated neuroendocrine carcinomas (NEC). While in gastroenteropancreatic NET a grading system based on proliferative activity (Ki-67 and mitotic index) is in place, mitotic index is integral part of the classification of pulmonary NET (Müller-Hermelink et al. 2004).

While these NET classifications were essential in obtaining worldwide standards and are able to predict clinical behaviour to some extent, they are far from reflecting the biological heterogeneity of NET. Novel technologies to analyse genomic and epigenomic characteristics have led to better understanding of this heterogeneous group of tumours. The actual knowledge of genetic, genomic and epigenomic data on pancreatic, small intestinal and pulmonary NET is summarized in this review as these are NET sites where recent research activity brought new findings in terms of molecular classification and they represent the majority of NET seen in an oncological setting (Dasari et al. 2017). The term neuroendocrine tumour (NET) is used hereafter to refer to gastroenteropancreatic and lung NET.

Genetics of NET

Genetic syndromes

The direct role of specific genes in tumour development is best seen in familial tumour syndromes. About 10% of pulmonary and GEP-NET occur in patients who have a cancer-predisposition syndrome. These syndromes include multiple endocrine neoplasia type 1 (MEN1) and von Hippel–Lindau syndrome (VHL), but also the less common neurofibromatosis type 1 (NF1) syndrome (Larsson et al. 1988, Wallace et al. 1990, Maher et al. 1991). Familial small intestinal NET seem to have a wider spectrum of very rare mutations (Sei et al. 2015, Dumanski et al. 2017), even if no gene mutation has been mechanistically proven to directly cause this disease.

Multiple endocrine neoplasia type 1 has high penetrance, over 90% by age 40 years, and it is found in 1–10 per 100,000 individuals. MEN1 patients present with tumours in the parathyroid glands (95%), the anterior pituitary (20–40%), in the endocrine cells of the pancreas/duodenum (40–80%) and of the lung (Brandi et al. 2001). The pancreatic and the thymic lesions (1–5% of MEN1 patients Teh et al. 1997) frequently develop into metastatic disease. The MEN1 gene on chromosome 11q13 includes 10 exons encoding the 610 amino acid menin protein. Menin is ubiquitously expressed and preferentially located in the nucleus, it has more than 40 interacting proteins, and it is involved in a large number of biological functions, such as DNA repair, chromatin modification, transcription, cell division, protein degradation, motility and adhesion (Agarwal et al. 2005, Balogh et al. 2006). By now, more than 1000 mutations have been recognized (Lemos & Thakker 2008), the majority leading to truncation of the protein. Most MEN1-associated tumours show somatic loss of the wild-type allele (loss of heterozygosity (LOH)) on chromosome 11q13, consistent with the role of MEN1 as a tumour suppressor gene (Anlauf et al. 2007, Perren et al. 2007).

Von Hippel-Lindau disease (VHL) is an autosomal dominant disorder with an incidence of 1/36,000 individuals (Maher et al. 1991). The onset results from inactivating mutations in the VHL tumour suppressor gene, which is located in 3p25, and it is involved in the oxygen-sensing pathway through regulation of hypoxia-inducible factors (Nordstrom-O’Brien et al. 2010). When VHL is truncated, the ubiquitination of HIF transcription factors cannot take place, and target genes controlling angiogenesis (VEGF and PDGFβ) and hypoxic metabolism result overexpressed (Iliopoulos et al. 1996, Siemeister et al. 1996). There are two different clinical manifestations of VHL, type 1 that arises from truncating mutations and type 2 that instead is the result of missense mutations (Chen et al. 1995). In general, VHL is characterized by increased risk of retinal and central nervous system (CNS) haemangioblastomas, pheochromocytoma (PCC), paragangliomas (PGL), renal clear cell carcinomas, renal cysts, pancreatic NETs (PanNET), pancreatic cysts and endolymphatic sac tumours. Exon 3 mutations seem to be associated with an increased risk of malignancy in pancreatic NET (PanNET) (Blansfield et al. 2007).

Neurofibromatosis type 1 (NF1) disease is a frequent genetic disorder linked to the development of multiple endocrinopathies and nervous system manifestations, of these, the most common are fibromatosus skin tumours,
café-au-lait spots, axillary and inguinal freckling, iris hematomas and optic gliomas (Basile et al. 2010, Relles et al. 2010, Pasmant et al. 2012). The prevalence of NF1 is about 1 in 3000 individuals. Approximately 40% of peripampillary duodenal somatostatinomas are associated with NF1 (Relles et al. 2010). PCCs and NET in other locations, including the pancreas can also develop (Perren et al. 2006). The NF1 tumour suppressor gene is located on chromosome 17q11.2, and it encodes the cytoplasmic protein neurofibromin that controls cellular proliferation by inactivating the p21 RAS protein (Martin et al. 1990). Loss-of-function mutations in NF1 lead to increased activity of the MAPK and PI3K-AKT-mTOR pathways (Brems et al. 2009).

**Hereditary small intestinal NET (SI-NET):** familial small intestinal NET (SI-NET) is a recently described condition, which is inherited in an autosomal dominant manner. To date, IPMK and MUTYH genes have been identified as causative germline mutations, but additional genes may be involved. SI-NET tend to be multifocal in patients with IPMK mutations. A prospective study conducted on 33 families (Sei et al. 2015), revealed a germline 4-bp deletion in the inositol polyphosphate multikinase (IPMK) gene on chromosome 10. This mutation was subsequently detected in all 11 affected individuals of this family but not in the other families, indicating that other genes are responsible for the onset of the disease. The IPMK mutation leads to a truncated protein and functional studies demonstrate reduced kinase activity and nuclear localization, which in turn reduces p53 activity and promotes cell survival (Sei et al. 2015). Independent studies are needed to validate the IPMK findings.

Recently, a monoallelic amino acid substitution (G396D) in the MutY DNA glycosylase gene (MUTYH) was reported as germline and somatic mutation in SI-NET (Dumanski et al. 2017). This protein is involved in oxidative DNA damage repair. This study points out the increasing importance of this specific pathway in NET.

Rare germline mutations in PanNET patients were also described in the base-excision-repair gene MUTYH (coupled with LOH) (5 individuals out of 98), in the BRCA2 gene (1 individual out of 98) and in the DNA damage repair gene CHEK2 (4 individuals out of 98) (Scarpa et al. 2017). All these PanNET were apparently sporadic and no data on penetrance of these germline mutations in PanNET are available. All tumours showed mutation profiles typical for repair gene deficiency, a phenotype only observed in this familial setting (Scarpa et al. 2017). Clinically, these new findings open the way to new treatment options for these PanNET patients, possibly including PARP inhibitors.

**Somatic genetic alterations**

Compared with other tumour types, NET are characterized by relatively few mutations and chromosomal aberrations per tumour (Fig. 1), except for the rare PanNET-associated with DNA repair mutations. The genes known from familial predisposition syndromes are also mutated in sporadic NET with the exception of VHL, which is rarely mutated in sporadic PanNET. The few genes so far associated with neuroendocrine tumorigenesis are members of important pathways involved in chromatin modification, telomere length maintenance, growth control, DNA damage response and cell metabolism (Figs 2 and 3).

**Pancreatic NET**

**Cell proliferation, metabolism and angiogenesis** Sporadic PanNET harbour mutations in genes encoding for members of the mTOR pathway (Jiao et al. 2011). Mutations of PTEN were found in 7.3% of PanNET and more recently mutations in TSC2, 8.8%; PIK3CA, 1.4% were also described (Perren et al. 2000, Jiao et al. 2011). TPS3 mutations are rare in PanNET.
and found mainly in G3 NEC and rarely G3 NET (Jiao et al. 2011, Scarpa et al. 2017).

A recent whole genome sequencing study in 98 PanNET confirmed mutations in mTOR pathway, 15% of cases. Notably, mutually exclusive mutations were found in PTEN (7.1%), TSC1 (2.0%), TSC2 (2.0%) and for the first time, in DEPDC5 (2.0%) (Scarpa et al. 2017). EWSR translocations also lead to an altered mTOR signalling (Scarpa et al. 2017).

A single study investigated 35 kinase genes commonly mutated in cancer in 36 primary PanNET and reported activating mutations in KIT in 2.7% of the samples (Corbo et al. 2012).

Gene expression and DNA repair

ATM (ATM serine/threonine kinase) was found mutated in 5.5% of PanNET (Corbo et al. 2012). ATM belongs to the class of caretaker tumour suppressor genes that defend genome integrity (Levitt & Hickson 2002). It is involved in multiple cellular processes that occur in response to DNA damage, including cell cycle check point control, DNA repair and apoptosis (Derheimer & Kastan 2010, Shiloh & Ziv 2013).

In 2013, WES of 10 sporadic insulinomas revealed a recurrent somatic T372R gain-of-function mutation in Ying Yang 1 (YY1) transcription factor gene. These results were validated on 113 additional tumours and 30% resulted affected (Cao et al. 2013).

YY1 regulates the mitochondrial function and insulin/insulin-like growth factor signalling (Cunningham et al. 2007, Blattler et al. 2012). T372R mutation increased the transcriptional activity of YY1 resulting in higher expression of its target genes like the mitochondrial genes IDH3A and UCP2 in mutated tumours (Cao et al. 2013).

Chromatin remodelling and telomeres maintenance

Mutations in genes encoding for proteins involved in chromatin remodelling are the most frequent in PanNET. Mutations of MEN1 and allelic loss of chromosome 11q are the most common genetic alterations, especially among non-functioning PanNET (NF-PanNET) (Moore et al. 2001, Perren et al. 2007).
Mutations of \textit{MEN1} have been found in 30% of sporadic NF-PanNET, 7% of insulinomas, 36% of gastrinomas, 67% of glucagonomas and 44% of VIPomas (Moore \textit{et al.} 2001). A recent study on microadenomas (considered precursors to PanNET in MEN1 patients) identified aberrant menin expression in 74% of the cases (14/19), suggesting that in this subset of cases, aberrant menin expression is a key initiator in PanNET tumorigenesis (Kloppel \textit{et al.} 2014, Esposito \textit{et al.} 2015, Hackeng \textit{et al.} 2016). \textit{Men1} encodes the transcription factor menin, which recruits the H3K4me3 histone methyltransferase mixed lineage leukaemia (MLL1) complex that plays an essential role in chromatin remodelling and gene expression (Agarwal \textit{et al.} 1999).

The first WES in PanNET reported, in 43% of the cases, mutations in \textit{DAXX} (death-domain-associated protein) or \textit{ATRX} (alpha thalassemia/mental retardation syndrome X-linked) (Jiao \textit{et al.} 2011). Both \textit{DAXX} and \textit{ATRX} are chromatin remodellers and are involved in the incorporation of the histone variant H3.3 at the telomeres and pericentric heterochromatin (Lewis \textit{et al.} 2010). Protein loss as well as mutations in \textit{DAXX} or \textit{ATRX} strongly correlates with the ALT phenotype in PanNET (Heaphy \textit{et al.} 2011). Patients with loss of \textit{DAXX} or \textit{ATRX} expression in PanNET have a poorer prognosis in surgical series (Marinoni \textit{et al.} 2014, Singhi \textit{et al.} 2017). Additionally, loss of \textit{DAXX} or \textit{ATRX} is associated with chromosome instability (CIN) possibly explaining the presence of chromosomal instability in a subset of more aggressive PanNET (Marinoni \textit{et al.} 2014, Pipinikas \textit{et al.} 2015, Scarpa \textit{et al.} 2017). \textit{DAXX/ATRX} loss is found almost exclusively in sporadic PanNET $>2$ cm (Marinoni \textit{et al.} 2014) and the same is true for \textit{DAXX/ATRX} mutations in MEN1 patients (de Wilde \textit{et al.} 2012). Such mutations are absent in sporadic and familial microadenomas. These results suggest that \textit{ATRX} and \textit{DAXX} loss as well as ALT activation are late events in PanNET tumourigenesis (de Wilde \textit{et al.} 2012). Recently Vinagre and coworkers described for the first time TERTp mutations in PanNET and PanNET-derived cell lines (Vinagre \textit{et al.} 2016). These mutations were prevalent in PanNET cases with hereditary component where they serve as an alternative mechanism of telomere length maintenance in an exclusive manner to ALT associated with \textit{DAXX/ATRX} loss. A missense mutation in a gene encoding H3 histone family 3A (H3F3A), also known as H3.3, was found in one insulinoma (1/10) (Cao \textit{et al.} 2013). H3.3 is important in chromatin modification, replication and repair of DNA, regulation of gene expression as well as maintenance of centromeres and telomeres. Very recently, with the histone modifier \textit{SETD2} (5 samples out of 98 mutated), an additional epigenetic modifier was described in PanNET (Scarpa \textit{et al.} 2017).

The epigenetic implications of these mutations are discussed in the epigenetic section.

The recent whole genome sequencing analysis on PanNET summarizes these findings in four commonly mutated pathways: DNA damage repair, chromatin remodelling, telomere maintenance and mTOR signalling (Scarpa \textit{et al.} 2017). A pivotal role in PanNET development was confirmed for the \textit{MEN1} gene, which is involved in all these pathways. Mutation signatures correspond to distinct tumour behaviour: notably mutations in the chromatin remodelling and mTOR pathways correlate with poor prognosis while tumours with an unaltered telomere length have a better outcome. The mutation spectrum seems to partially reflect molecular subtype of PanNET. In a comparative analysis between a mouse model (Rip1TAG2) and human PanNET, three main biological subgroups of tumours have been described based on transcriptomic analysis: well-differentiated islet/tumour (IT), less differentiated PanNET with more...
frequent metastasis (metastasis-like primary, MLP) and intermediate MEN1-like tumours. Mutation in the mTOR pathway (TSC2/PTEN) and in the DNA repair pathway gene ATM were equally distributed between the MLP and IT tumours. Mutations in chromatin remodelling genes were instead enriched in MLP and in the MEN1-like tumours (MEN1 mutated) with an intermediate phenotype (Sadanandam et al. 2015). These results indicate that different mutational subgroups contribute partially to transcriptomic and prognostic groups of PanNET.

However, the correlations are not very strong and the clinical implication of these subgroups still need to be better defined, especially in respect to their value to predict response to specific therapies.

**Small intestinal NET**

The first whole-exome sequencing (WES) on 48 SI-NET was performed in 2013 (Banck et al. 2013). In this study, mutations in several cancer genes were found but none was recurrently altered. Mutation rate of SI-NET genomes was low, with an average of 0.1 somatic single-nucleotide variants per 10⁶ nucleotides in the exome (Fig. 1). The SI-NET mutation rate was similar to lung carcinoids (Greenman et al. 2007) and PanNET and was lower than mutation rates in other cancers (Stransky et al. 2011, Barbieri et al. 2012, Cancer Genome Atlas N 2012a,b, Ellis et al. 2012, Imielinski et al. 2012). Carrying on WES, therapeutically relevant candidate alterations were found.

**Cell proliferation**

Francis and coworkers (Francis et al. 2013) found in a larger cohort of 180 SI-NET including 48 cases from Banck and coworkers heterozygous frame shift mutations of CDKN1B in 14 of 180 tumours (8%). CDKN1B encodes the p27 protein, which regulates cell cycle progression (Figs 2 and 3) (Polyak et al. 1994). Cell cycle deregulation may thus be one potential mechanism in SI-NET tumorigenesis. A further study aimed to re-sequence a clinically well-characterized cohort consisting of 362 SI-NET samples from 200 patients for CDKN1B mutations and to investigate possible genotype–phenotype correlations (Crona et al. 2015). This study confirmed CDKN1B as a potential tumour suppressor gene in SI-NET. 8.5 % (17/200) of the patients had CDKN1B mutations. Interestingly, within tumour lesions, the majority of the investigated patients show heterogeneity for CDKN1B mutations. Because of this, it was not possible to identify patients with CDKN1B mutated tumours as having a unique clinical phenotype. Future studies will have to define the selective advantages acquired in SI-NET through inactivation of CDKN1B. A more recent study confirmed CDKN1B mutations in 8% of samples (Karpathakis et al. 2016).

**Pulmonary NET**

**Chromatin remodelling**

Somatic MEN1 gene mutations were found in approximately 13–18% of sporadic pulmonary carcinoids (Debelenko et al. 1997).

In 2014, the whole exome of 69 pulmonary carcinoids was sequenced, revealing the most frequently mutated genes, in addition to MEN1, in this malignancy (Fernandez-Cuesta et al. 2014) (Figs 2 and 3). All mutations were found in genes acting in chromatin remodelling pathways. The mean somatic mutation rate was of 0.4 mutations per megabase (Mb), which again is much lower than the rate observed in adenocarcinomas (AD), squamous (SQ) and small-cell lung cancer (SCLC) (Fig. 1). The only recurrently mutated genes were MEN1, ARID1A and EIF1AX. In general, 40% of the cases carried mutually exclusive mutations in genes that are encoding for histone covalent modifiers: MEN1, the menin-binding protein PSIP1 (in six cases mutually exclusive with MEN1), members of the Polycomb repressive histone H3K27 methyltransferase complexes (CBX6, EZH1 and YY1), histone lysine demethylases (KDM4A, PHF8 and JMJD1C), histone lysine methyltransferases (SETD1B, STDB1 and NSD1) and histone acetylation modifiers (BRWD3 and HDAC5). Mutations were found also in ATP-dependent chromatin remodelling SWI/SNF complex members: ARID1A that provide specificity to the complex, the core subunits SMARCA1, SMARCA2 and SMARCA4 that are important for the ATPase activity of the complex, as well as the subunits ARID2, SMARCC2, SMARCB1 and BCL11A. Sister chromatid cohesion pathway was also recurrently affected by mutations and the following genes were found mutated: the cohesion subunit STAG1, the cohesion loader NIPBL, the ribonuclease and miRNA processor DICER and ERC6L, involved in sister chromatid separation. In addition, other statistically significant mutated genes were EIF1AX, SEC31A, WDR26 and the E3 ubiquitin ligase HERC2. In this study, TP53 and RB1 genes, commonly mutated in SCLC, were found mutated only in 2 atypical carcinoids. These findings confirm the theory that pulmonary carcinoids and SCLC are two distinct specimens of tumours.
Chromosomal rearrangement

The most frequent chromosomal rearrangement found in pancreatic, small intestinal and pulmonary NET are summarized in Table 1.

Pancreatic NET

A number of comprehensive studies have been carried out, employing both array comparative genomic hybridization (aCGH) as well as single-nucleotide polymorphism (SNP) approaches in order to elucidate cytogenetic aspects of PanNET.

Capurso and coworkers and Jonkers and coworkers reviewed extensively CGH of PanNET (Jonkers et al. 2007, Capurso et al. 2012). Non-functional PanNET present the highest rate of genomic aberrations, and they frequently show loss of chromosome 11q and they frequently show loss of chromosome 11q (containing the genes MEN1, BRCA2 and ATM), 6q and 11p, as well as gains in 17q (NEU/ERB2), 7q and 20q (AURKA, cMET). Malignant insulinoma follow the NF-PanNET for a number of genomic aberrations, including 17q and loss of 6q. Benign insulinomas and gastrinomas present the lowest amount of changes. In insulinomas, early events are gain of chromosome 9q and loss of 22q along with 11q, telomeric loss is recognized during disease progression. In gastrinomas, common chromosomal aberrations are 3p (loss) and 9p (gain). Loss of heterozygosity studies in PanNET indicate loss of 22q12.1 (75%), 3p23 (74%), 11q13 (67%; Men1), 6p22 (62%), 10q23 (50%; PTEN). Ohki and coworkers recently reported that the genomic region of the PHLD3A gene (1q31) undergoes LOH in 75% of PanNET, and they could correlate this event with disease progression and adverse prognosis (Ohki et al. 2014). PHLD3A locus undergoes methylation in addition

<table>
<thead>
<tr>
<th>Alteration</th>
<th>PanNET Frequency (%)</th>
<th>SI-NET Frequency (%)</th>
<th>Lung NET Frequency (%)</th>
<th>Putative genes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>−1q</td>
<td>10–24</td>
<td></td>
<td></td>
<td>HHPT2, MDA7, PHLD3A</td>
<td>Capurso et al. (2012)</td>
</tr>
<tr>
<td>−1p</td>
<td>3–28</td>
<td></td>
<td></td>
<td>HP73, CDKN2C, RUNX3</td>
<td>Capurso et al. (2012)</td>
</tr>
<tr>
<td>−3p</td>
<td>19–27</td>
<td></td>
<td></td>
<td>VHL, hMLH1, RARβ1, CTNNB1, RASSF1A, RYBP</td>
<td>Capurso et al. (2012)</td>
</tr>
<tr>
<td>−6q</td>
<td>3–70</td>
<td></td>
<td></td>
<td>AIM1</td>
<td>Capurso et al. (2012)</td>
</tr>
<tr>
<td>−10q</td>
<td>3–26</td>
<td></td>
<td></td>
<td>MGMT, PTEN</td>
<td>Capurso et al. (2012)</td>
</tr>
<tr>
<td>−11q</td>
<td>13–39</td>
<td>28</td>
<td></td>
<td>MEN1, PLCB3, SDHD, TSG11, HHPT, BRCA2, ATM, CASP11, CASP5</td>
<td>Capurso et al. (2012), Swarts et al. (2012)</td>
</tr>
<tr>
<td>−11p</td>
<td>3–34</td>
<td></td>
<td></td>
<td>WT1</td>
<td>Capurso et al. (2012)</td>
</tr>
<tr>
<td>−18</td>
<td>3–34</td>
<td>61–89</td>
<td></td>
<td>SMAD2, SMAD4, DPC4</td>
<td>Andersen et al. (2009), Cunningham et al. (2011), Francis et al. (2013), Kim et al. (2008), Kulke et al. (2008)</td>
</tr>
<tr>
<td>+4</td>
<td>20–33</td>
<td></td>
<td></td>
<td></td>
<td>Andersen et al. (2009), Cunningham et al. (2011), Francis et al. (2013), Kim et al. (2008), Kulke et al. (2008)</td>
</tr>
<tr>
<td>+5</td>
<td>13–28</td>
<td></td>
<td></td>
<td></td>
<td>Andersen et al. (2009), Cunningham et al. (2011), Francis et al. (2013), Kim et al. (2008), Kulke et al. (2008)</td>
</tr>
<tr>
<td>+7q</td>
<td>12–47</td>
<td></td>
<td></td>
<td>HGF, C-MET</td>
<td>Capurso et al. (2012)</td>
</tr>
<tr>
<td>+7p</td>
<td>6–37</td>
<td></td>
<td></td>
<td>ERBB1</td>
<td>Capurso et al. (2012)</td>
</tr>
<tr>
<td>+9q</td>
<td>12–43</td>
<td></td>
<td></td>
<td>VAV2, CDK9, cABL, NOTCH1, LMX1B</td>
<td>Capurso et al. (2012)</td>
</tr>
<tr>
<td>+9p</td>
<td>6–29</td>
<td></td>
<td></td>
<td>JAK2, oncogene ovr, RAGA, CDKN2A</td>
<td>Capurso et al. (2012)</td>
</tr>
<tr>
<td>+14</td>
<td>20–30</td>
<td></td>
<td></td>
<td></td>
<td>Andersen et al. (2009), Cunningham et al. (2011), Francis et al. (2013), Kim et al. (2008)</td>
</tr>
<tr>
<td>+17q</td>
<td>5–57</td>
<td></td>
<td></td>
<td>ERBB2</td>
<td>Capurso et al. (2012)</td>
</tr>
<tr>
<td>+20</td>
<td>17–34</td>
<td></td>
<td></td>
<td></td>
<td>Andersen et al. (2009), Cunningham et al. (2011), Francis et al. (2013), Kim et al. (2008), Kulke et al. (2008)</td>
</tr>
<tr>
<td>+20q</td>
<td>6–43</td>
<td></td>
<td></td>
<td>AURKA</td>
<td>Capurso et al. (2012)</td>
</tr>
</tbody>
</table>

Only alterations with frequencies higher than 20% are included in the table.

*Atypical and typical carcinoids.
to LOH. The most recent study describes four groups of PanNET patients regarding copy number changes (Scarpa et al. 2017): Group one had a recurrent pattern of whole chromosomal loss (RPCL) (chromosomes 1, 2, 3, 6, 8, 10, 11, 15, 16 and 22) and was enriched in G2 tumours. Group two had a limited number of events mainly affecting chromosome 11. Group three, polyploid tumours, showed gains of whole chromosome and had also the highest somatic mutation rate. Group four was characterized by aneuploidy and whole chromosome gains. Recurrent broad region of loss contained MEN1 and CDKN2A genes and focal losses highlighted EYA1, FMBTI and RABGAP1L as potential tumour suppressor genes. Amplified regions included instead PSPN (activating PIK3CA) and ULK1 (mTOR-regulated autophagy). Inactivation of tumour suppressor occurred through chromosomal rearrangements for MTAP, ARID2, SMARCA4, ML3, CDKN2A and SETD2 genes. Additionally, the authors revealed a previously undescribed mechanism of oncogenic driver in PanNET: the fusion events of EWSR1 with BEND2 and FLI1. These alterations may lead to mTOR signalling activation and open the way to identification of new biomarker for selecting mTOR inhibitor treatment.

Interestingly, RPCL strongly associated with ALT, confirming that ALT activation and chromosomal instability belong to the same mechanism of PanNET formation and development. On the other hand, genome catastrophe and EWSR1 were associated with short telomeres supporting the hypothesis that telomere exhaustion is involved in chromothripsis.

**Small intestinal NET**

Karpathakis and coworkers recently performed a genome-wide molecular profiling of SI-NET, analysing genomic, epigenomic and transcriptomic characteristics (Karpathakis et al. 2016). The authors could identify three groups correlating with significantly different progression-free survival: One group harboured chr18 LOH only (18LOH group, 55% of tumours), another no large copy number alterations (No CNV group, 19%) and the third one harboured multiple copy number alterations (including gain of 4, 5, and 20; multi-CNV group, 26%). Of note, tumours that harboured CDKN1B mutations were located within the first group (chr18 LOH). A difference in the progression-free survival was identified between the three subgroups with the 18LOH group having superior progression-free survival when compared with the other two groups.

Looking at SCNAs, the most common in SI-NET is loss of chromosome 18, first described in 2001 (Kytola et al. 2001, Lollgen et al. 2001). It occurs in more than 60% of tumours (Wang et al. 2005, Kulke et al. 2008, Andersson et al. 2009, Banck et al. 2013, Hashemi et al. 2013). Relevant genes at 18q21.1 include SMAD2 and SMAD4, but to date, sequencing of these genes has not revealed any mutations (Lollgen et al. 2001, Kulke et al. 2008). Other common losses in SI-NET occur on chromosomes 11q, 16, 9, 13 and 3p (Kim et al. 2008, Kulke et al. 2008, Andersson et al. 2009, Cunningham et al. 2011). Different putative candidate tumour suppressor genes have been identified in these regions, like p16/CDKN2A on 9p, RYBP on 3p, SDHD, CASP1, 4 and 5 on 11q and CDH1 on 16q (Kim et al. 2008, Kulke et al. 2008, Andersson et al. 2009, Cunningham et al. 2011). Andersson and coworkers demonstrated a strong correlation between gain of chromosome 14 and poor survival (Andersson et al. 2009). Kulke and coworkers observed a local amplification of a locus in chromosome 14q, a small region in this locus encompasses a control element known to influence the expression of the antiapoptotic protein DAD1. Immunostaining of DAD1 confirmed its overexpression in small bowel carcinoid tumour cells, compared with normal mucosa or surrounding stroma. Other limited amplification areas included the locus of ORF4A5 (olfactory receptor) and PRKCA (protein kinase C alpha) (Kulke et al. 2008). Andersson and coworkers compared CNAs between primary tumours and liver metastases and the average number of CNAs per tumour was twice as high in liver metastases (9.5, range 2–22) as in primary tumours (4.3, range 1–18) (Andersson et al. 2009). This underlines the accumulation of ‘genomic changes’ during tumour progression.

From genomic profile studies, it was possible to describe at least two different groups, one characterized by loss of chromosome 18 as an early event, followed by additional losses later and another one characterized by the presence of intact chromosome 18 but with clustered gains on chromosomes 4, 5, 7, 14 and 20 (Kulke et al. 2008, Andersson et al. 2009).

**Pulmonary NET**

Swarts and coworkers performed a meta-analysis of specific genomic alterations in lung NET integrating data...
from CGH data or from other cytogenetic approaches for individual tumours (Swarts et al. 2012). Chromosomal alterations in typical carcinoids (TC) (on average 2.8 aberrations per tumour) and atypical carcinoids (ACs) (6.1) were much rarer if compared with SCLC (18.8) and LCNECs (13.7). Recurrent chromosomal losses were found on 11q and 13q, known for the location of MEN1 (11q22-23 and 11q24) and the tumour suppressor gene retinoblastoma (RB; 13q14).

**Epigenetic modification**

Cancer cells are characterized by a complex interplay between genetic and epigenetic abnormalities that drives the evolution of each malignancy. The very low mutations rate observed in NET compared to other tumour entities suggests that other mechanisms, such as epigenetic changes could be involved in NET development and progression.

Epigenetics literally means ‘above’ or ‘on top of’ genetics. It refers to external modifications to DNA, which do not alter the DNA sequence, but affect chromatin structure influencing gene expression and genomic stability. Epigenetic changes are conserved and propagated to daughter cells in divisions, enabling cancer cells epigenetic progression. Epigenetic changes consist of DNA methylation and histone modification. Both are highly dysregulated in cancer including NET and contribute to the tumour evolution.

**DNA methylation**

DNA methylation consists in the addition of a methyl group to a cysteine by DNA methyltransferase enzymes (DNMT1 and DNMT3). This modification affects the chromatin structure organization. Highly methylated promoters are not accessible to the transcriptional machinery, and the genes are silenced. Vice versa, hypomethylation of promoters induces corresponding gene expression. DNA methylation not only regulates gene expression but is also important for the maintenance of genomic stability.

**Histone modifications**

Post-translational covalent histone modifications regulate accessibility of genes to transcription factors by formation of permissive or repressive chromatin structure, thereby regulating gene expression (Chi & Bernstein 2009). Specific enzymes are responsible for the different modifications on specific histones, which include acetylation, lysine methylation and phosphorylation. DNA methylation status can affect the methylation states on accompanying histones in chromatin and vice versa. Specific DNA modification are influenced by the histone lysine methylation state (Rose & Klose 2014). Indeed, DNA methylation is associated with specific histone modifications, in particular with high levels of H3K9 three-methylation and absence of histone H3K4 three-methylation. Lately, aberrant levels of specific histone variants or mutations in genes encoding for histones themselves have been reported in several type of cancers including NET (Jiao et al. 2011, Cao et al. 2013, Fernandez-Cuesta et al. 2014).

The principal differentially methylated regions in pancreatic, small intestinal and pulmonary NET are summarized in Table 2.

**Pancreatic NET**

Epigenetic dysregulation observed in PanNET includes aberrant DNA methylation of specific regions and mutations in genes such as DAXX, ATRX and MEN1, which are involved in chromatin structure remodelling, histone modification and DNA methylation. Historically, methylation changes in PanNET have been assessed without taking into account the genetic background. However, since the discovery of DAXX and ATRX mutations, their contribution in determining specific epigenetic patterns seems very likely.

In the following sections, we first discuss DNA methylation changes observed in PanNET regardless the genetic background and second the direct effect induced by DAXX, ATRX and MEN1 on the epigenetic status.

**DNA methylation** Impairment in DNA methylation has been reported in PanNET. Most of the studies report aberrant methylation of one single gene or few candidates. Several studies indicated RASSF1A as one of the most hypermethylated genes in PanNET and more frequently in metastatic tumours compared to non-metastatic ones. Interestingly, RASSF1A promoter was found methylated in normal pancreas as well, although to a lower level (Dammann et al. 2003, Malpeli et al. 2011). We found VHL promoter (VHL-associated gene) hyper-methylation in a subset of PanNET in correlation with intra-tumoural hypoxia and poor prognosis (Schmitt et al. 2009). In another study conducted on 46 pancreatic NET methylation of RUNX3, MGMT, APC and
HIC1 was frequently found. CIMP phenotype defined as 2 or more genes methylated was found in 80% of the tumours (Arnold et al. 2007). CDKN2a (p16) promoter is methylated in 40% of PanNET, and it is associated with early tumour recurrence and reduced overall survival (House et al. 2003). Hypermethylation of the promoter of TIMP-3 (tissue inhibitor of metalloproteinase-3) gene was found in 44% of PanNET in a study performed on 18 PanNET in correlation with protein loss. TIMP-3 alterations were significantly more frequent in metastatic setting (Wild et al. 2003). Promoter hyper-methylation of MHL1 and hyper-methylation of IGF2 DMR2 were found exclusively in insulinomas in association with increased malignancy. (Dejeux et al. 2009). MGMT promoter methylation occurs in PanNET as well (Schmitt et al. 2014, Walter et al. 2015).

Hypo-methylation of LINE1 (long interspersed element 1) and ALU sequences compared to normal tissues have been reported as well (Choi et al. 2007). LINE1 hypomethylation significantly correlated with poor prognosis and advanced tumour stage in PanNET (Stefanoli et al. 2014). Interestingly, in the same study, the authors found that DAPK1, TIMP3, PAX5, HIC1, CADM1, PYCARD, VHL, RARB and WT1 promoters were significantly hyper-methylated in late-stage tumours and in tumours with poor prognosis (Stefanoli et al. 2014). However, the majority of the tumours did not show simultaneous global hypomethylation and CpG hypermethylation suggesting that in PanNET the two mechanisms are independent.

**Table 2** DNA methylation in pancreatic, small intestinal and lung NET.

<table>
<thead>
<tr>
<th>Function</th>
<th>Non-functioning PanNET</th>
<th>Insulinoma</th>
<th>SI-NET</th>
<th>LungNET</th>
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<td></td>
<td>Genes</td>
<td>Genes</td>
<td>Genes</td>
<td>Genes</td>
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<tr>
<td>Cell cycle</td>
<td>RASSF1A</td>
<td>RASSF1A</td>
<td>RASSF1</td>
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<td></td>
<td>CDKN2A</td>
<td>CHFR</td>
<td>p15</td>
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<tr>
<td>DNA repair</td>
<td>MGMT</td>
<td>MHL1</td>
<td>APOBEC3C</td>
<td>MGMT</td>
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<tr>
<td>Cell metabolism</td>
<td>ESR1</td>
<td>TCEB3C</td>
<td>GIPR</td>
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<td></td>
<td>RARB</td>
<td>CXX5S</td>
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<tr>
<td>Cell growth</td>
<td>HIC1</td>
<td>IGF2</td>
<td>SETBP1</td>
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<td></td>
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<td>MAPK4</td>
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<td>Cell death</td>
<td>DAPK1</td>
<td>Pycard</td>
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<tr>
<td>Cell development</td>
<td>PAX5</td>
<td>RUNX3</td>
<td>RUNX3</td>
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<td></td>
<td>WT1</td>
<td>MBD1</td>
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<tr>
<td>Gene expression</td>
<td>RUNX3</td>
<td>RUNX3</td>
<td></td>
<td></td>
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<tr>
<td>Angiogenesis/hypoxia</td>
<td>VHL</td>
<td>TIMP3</td>
<td>CTNNB1</td>
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<tr>
<td>Invasion</td>
<td>APC</td>
<td>CADM1</td>
<td>LAMA3</td>
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<tr>
<td>Non-coding</td>
<td>ALU</td>
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<td></td>
<td>LINE1</td>
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**DAXX and ATRX mutations** Mutations in the chromatin remodeller DAXX and ATRX occur in 40% of sporadic PanNET (Jiao et al. 2011). Potential mechanisms involved in PanNET development associated with DAXX/ATRX loss impacting on epigenetic status are depicted in Fig. 4: DAXX and ATRX form a complex, which
mediates the deposition of the H3.3 variant in repeated genomic sequences such as CpG islands, telomeres and peri-centromeric regions (Lewis et al. 2010). DAXX/ATRX complex regulates the H3K9me3 levels, thus ensuring a silent and condensed chromatin status in heterochromatin regions including telomeres. Additionally, ATRX has the ADD domain (ATRX-DNMT3-DNMT3L) as DNMT3A for the H3 binding, while DAXX binds and mediates DNMT1 recruitment at specific promoters such as RASSF1A and RELB regulating gene expression (Puto & Reed 2008, Iwase et al. 2011, Zhang et al. 2013). Specific methylation profiles of DAXX and ATRX mutated tumours have been recently described. Differences in DNA methylation in DAXX mutated tumours compared to control seem to be more pronounced (Fig. 4) (Pipnikas et al. 2015).

Yet, the tumour pathways associated with DAXX and ATRX loss are not well understood. Epigenetic changes may represent the main driver in the progression of these PanNET (Fig. 4). Also ALT activation, which highly correlates with DAXX or ATRX loss in PanNET, may be induced by increased recombination at telomeric sites due to hypo-methylation of the telomeric region or H3.3 deposition impairment, both resulting in an open chromatin structure (Fig. 4). Long interspersed nuclear elements (LINE1) are the most abundant mobile DNAs in the human genome. Hypo-methylation of these mobile DNAs causes their transcriptional activation and has been found in many types of cancer. Hypo-methylation of the mobile DNA can cause genomic instability and disruption of expression of the adjacent gene as well (Kitkumthorn & Mutirangura 2011).

Similarly, CIN observed in DAXX/ATRX negative tumours maybe epigenetically induced. LINE1 hypo-methylation has been observed in PanNET with high CIN, suggesting that hypo-methylation of repetitive elements may be the reason for CIN in PanNET (Marinoni et al. 2017). Further studies are needed in order to dissect the molecular mechanisms associated with DAXX and ATRX loss in PanNET development.

**Men1** Men1 is the most frequently mutated gene in PanNET and encodes the transcription factor menin. Menin recruits the H3K4me3 histone methyltransferase complex, which is crucial for regulating gene expression and chromatin remodelling (Agarwal et al. 1999). Studies on mice with heterozygous loss of Men1 report upregulation of genes involved in histone methylation (MLL1) and histone acetylation (CHD4) or chromatin modification (SMARCC2, HIST4H4, SUV420H2, CBX6, HLFX, HDAC9, HDAC10) (Mould et al. 2007, Lejonklou et al. 2012). A decrease in global methylation level of histone H3K9me3 and H3K4me3 is observed in Men1(−/−) murine cells (Agarwal & Jothi 2012, Yang et al. 2013). In Men1(−/−) mice histone H3K27me3 levels are enriched, with a concomitant decrease in H3K4me3 within the promoters of several genes. Notably, among the dysregulated genes the insulin-like growth factor2 mRNA binding protein (IGF2BP2) is downregulated in Men1(−/−) due to a decreased of H3K4me3 levels in the promoter regions. Interestingly knock-out of the histone de-methylase enzyme RBP2 (KDM5) reduced tumour burden in Men1(−/−) mice and partially restored gene expression including IGF2BP2 (Lin et al. 2011, 2015). KDM5 inhibitors are in development and may represent an attractive option for the treatment of MEN1-associated NET. Another study reported hyper-methylation in
four CpG islands of the IGF2 gene in Men1(−/−) mice with consequent IGF2 overexpression. IGF2BP2 inhibits translation of Igf2 during embryonic development. Whether it has similar functions in adults is unknown (Fontaniere et al. 2006, Lin et al. 2015). Downregulation of IGF2BP2 and IGF2 overexpression in Men1(−/−) might be an example of fine adjustment via different epigenetic mechanisms.

Recently, menin and DAXX were shown in vitro in rodent cells to cooperate to enhance H3K9me3 levels at the promoter of Membrane metallo-endopeptidase (MME), inhibiting its expression (Feng et al. 2016).

**Pulmonary NET**

Similar to PanNET pulmonary NET display beside DNA methylation aberration, frequent mutations in genes encoding for proteins directly involved in the epigenetic status maintenance (Figs 2 and 3).

DNA methylation of RASSF1 has been found in pulmonary NET in correlation with tumour grade but not with mRNA levels (Pelosi et al. 2010). Aberrant methylation at the 5' end of the cell cycle regulator, p15 (INK4b) gene was observed in 15% of NE lung tumours, in a region where methylation did not occur in control and associated normal lung. However, no correlation with protein status could be assessed (Chaussade et al. 2001).

Recently, WES on 44 pulmonary carcinoids revealed in 80% mutations in several genes involved in chromatin structure organization, indicating a prominent role of epigenetic dysregulation in the biology of these tumours (Figs 2 and 3). The high prevalence of mutations in chromatin remodelling genes suggests that these mutations are crucial to drive tumorigenesis in a precisely defined spectrum of required cellular pathways (Fernandez-Cuesta et al. 2014). Previous studies already reported impairment in specific histone 4 modification levels. Additionally, EZH2 (histone methyltransferase enhancer), which is a component of a chromatin remodelling complex that mediates lysine 27 methylation of histone H3 (H3K27), is overexpressed in high-grade neuroendocrine tumours of the lung (Findeis-Hosey et al. 2011). Genome-wide epigenetic analysis on pulmonary NET has not yet been reported.

**Small intestinal NET**

Global hypo-methylation seems to be a general feature of gastrointestinal NET. Hypo-methylation of LINE1 and ALU sequences was found in ileal NET (Choi et al. 2007).

Interestingly, LINE1 hypo-methylation correlated with tumour size, RASSF1A promoter hyper-methylation, loss of chromosome 18 and MGMT promoter methylation. More recently, LINE1 hypo-methylation has been described in 80% of small intestine tumours but no correlation with grade and tumour size was found (Stricker et al. 2012).

In a study including 33 ileal carcinoids promoter methylation of RASSF1A and CTNNB1 was found more frequently in metastatic tumours. Interestingly, Western blotting analysis of matched primary and metastatic tumours showed a decreased expression of RASSF1A and b-catenin in the metastases, suggesting a role for CTNNB1 and RASSF1A in tumour progression and metastasis formation. However, IHC on the same samples did not reveal any expression differences of these two proteins between primary tumours and metastases (Zhang et al. 2006).

Another study confirmed hyper-methylation of RASSF1A promoters in distant metastasis vs primary tumours but not CTNNB1. No correlation with RASSF1A methylation status and clinical behaviour was found (Fotouhi et al. 2014).

The TCEB3C (Transcriptional Elongation Factor B polypeptide 3C) that encodes for Elongin A3 involved in the RNA polymerase transcription elongation seems to be epigenetically silenced in SI-NET. TCEB3C is the only imprinted gene on chromosome 18, which is often lost in SI-NET; thus, patients only display one copy of the gene. TCEB3C promoter was found hyper-methylated in all 14 SI-NET analysed. Interestingly, treatment of primary tumours cells as well as SI-NET cell lines with the de-methylating agent decitabine and the histone methyltransferase inhibitor 3-deazaneaplacolin A induced TCEB3C expression, suggesting an epigenetic regulation of this gene in SI-NET (Edfeldt et al. 2014).

Genome-wide methylation analysis on 10 primary SI-NET and 10 matched mesenteric lymph node metastases revealed a general decrease in methylation levels in the metastases compared to the primary tumours. The tumour suppressors RUNX3, TP73 and CHFR were highly methylated in the majority of the SI-NET. Additionally, at chromosome 18q21-qter, SETBP1, ELAC1, MBD1, MAPK4 and TCEB3C were highly methylated in all SI-NET included in the cohort. Methylation of AXL, CRMP1, FGFS, CXXC5 and APOBEC3C genes was significantly different in primary tumours compared to metastases. Unsupervised hierarchical clustering of the samples identified three distinct clusters. Interestingly, the tumours with the highest methylation index included the ones with an aggressive phenotype (Verdugo et al. 2014).
A second genome-wide methylation study was performed on 97 SI-NET including both primary and liver metastasis (Karpathakis et al. 2016). Integration analysis with methylation and copy number variation delineates 3 different clusters of tumours. Patients with low CIN, loss of chromosome 18 and mutation in CDKN1B had better disease outcome than patients showing high CIN and no chromosome 18 loss. Hyper-methylation of the gastric inhibitory polypeptide receptor (GIPR) gene body and hypo-methylation of the promoter were associated with increased gene expression and metastasis formation. The epigenetic dysregulation of GIPR seems to be specific for SI-NET, and it is not found in other gastric malignancies.

Interestingly, epigenetic silencing of several genes on chromosome 18 including laminin alpha 3 (LAMA3), serpin peptidase inhibitor clade B member 5 (SERPINB5) and factor receptor superfamily member 11a NFkB activator (RANK or TNSFRSF11A) were found, suggesting these being a possible second hit upon chromosome 18 loss (Karpathakis et al. 2016).

The same authors in a follow-up study compared methylation and expression profiles of primary SI-NET and liver metastases (Karpathakis et al. 2017). They found a progressive change in methylation and respective gene expression between the primary and metastatic specimens in the same genes already reported to be epigenetically dysregulated (Karpathakis et al. 2016). These findings suggest that progression to metastasis in SI-NET could be driven by epigenetic dysregulation.

Very few studies have investigated histone modification levels in SI-NET. Increased levels of demethylation of histone H3 at lysine 4 (H3K4Me2) were found in 16 SI-NET, but further studies are needed to confirm these findings and to understand the molecular mechanisms behind it (Magerl et al. 2010).

Clinical implication

Epigenetics of NET may have important clinical implications. Methylation profiles can distinguish tumour subtypes, which potentially will allow better prediction of response to chemotherapeutic agents and of survival. The MGMT promoter is one of the most frequently hyper-methylated ones in PanNET. MGMT (O6-methylguanine-methyltransferase) is an important enzyme of DNA repair. MGMT promoter methylation has a predicting power in glioma patients treated with temozolomide. Similarly to gliomas, MGMT promoter methylation seems to predict for better therapy response to the DNA alkylating agent temozolomide in PanNET patients; however, larger and randomized clinical studies should be performed to confirm these findings (Schmitt et al. 2014, Walter et al. 2015).

Epigenetics represents a very appealing target in cancer treatment, because it can be modified. Several ‘epigenetic drugs’ are currently in clinical trials while others are already used for the treatment of other cancer types. DNA methyl-transferase inhibitor such as Azacitidine and Decitabine as well as histone deacetylases (HDAC) inhibitors such as Vorinostat and Belinostast are FDA approved for treatment of leukaemia.

DNA methylases inhibitor and HDAC inhibitor have been extensively used in vitro in both PanNET and small intestine cell lines showing results in terms of reducing cell viability and increasing gene expression (Baradari et al. 2006, Zhang et al. 2006, Habbe et al. 2007, Alexander et al. 2010, Arvidsson et al. 2016). Interestingly, Decitabine increased the expression of SSTR2 and the Ga-DOTATOC uptake in BON1 tumour-bearing mice, indicating a possible therapy implication in re-expression the target for somatostatin receptor-based radiotherapy (Taelman et al. 2016). However, despite the promising results in pre-clinical models, trials with approved epigenetic drugs in NET have shown very limited results. This failure may be due to the fact that, patients have not been stratified and selected according to their epigenetic profiles. The molecular characterization of the epigenetic changes in each tumour may indeed indicate which patient may profit from a specific drug as well as reveal crucial therapy targets. Retrospective studies for understanding epigenetic profiles will be the basis for better indication of specific epigenetic modifiers for future specific treatments.

MicroRNA (miRNA) expression profiles

Pancreatic NET

There are only few studies investigating the effect of microRNAs (miRNA) in PanNET. Roldo and coworkers showed upregulation of miR-103 and downregulation of miR-155 in different pancreatic cancers compared to normal tissue by global miRNA expression analysis (Roldo et al. 2006). In insulinoma, miR-204 was expressed in correlation with insulin expression. miR-21 expression was associated with liver metastatic disease and tumour grade (i.e. Ki-67 index), similar to what was observed in pulmonary carcinoids (see below). A recent study identified miRNA signatures in PanNET in tissue and serum. In tumoural tissue, miR-262 expression correlated with Ki67 score while miR-210 below). A recent study identified miRNA signatures in PanNET in tissue and serum. In tumoural tissue, miR-262 expression correlated with Ki67 score while miR-210 expression between the primary and metastatic specimens of SI-NET showed upregulation of miR-103 and downregulation of miR-155 in different pancreatic cancers compared to normal tissue by global miRNA expression analysis (Roldo et al. 2006). However, despite the promising results in pre-clinical models, trials with approved epigenetic drugs in NET have shown very limited results. This failure may be due to the fact that, patients have not been stratified and selected according to their epigenetic profiles. The molecular characterization of the epigenetic changes in each tumour may indeed indicate which patient may profit from a specific drug as well as reveal crucial therapy targets. Retrospective studies for understanding epigenetic profiles will be the basis for better indication of specific epigenetic modifiers for future specific treatments.
were more abundant in patients compared to healthy volunteers and miR-193 was upregulated in both serum and PanNET tissue.

In the Rip1Tag2 mouse model of insulinoma, specific miRNA signatures reflected the multistage progression of the disease. Notably miRNA profiles cluster: metastasis, tumours and rare aggressive tumours with metastasis features (metastasis-like primary tumours) (Olson et al. 2009). miRNA profiling and analysis of the human PanNET dataset generated by Roldo and coworkers showed clustering in three distinct groups based on a signature of 30 miRNAs (Roldo et al. 2006). A cross-species analysis between the mouse MLP signature and the human profiles identified 8 miRNAs (miR-137, miR-132, miR-181a2, miR-181a1, miR-23b, miR-27b, miR-24-2 and miR-24-1) being highly expressed in two of the human clusters. miR-137 the most enriched miRNA in the MLP group was the highest expressed in one human cluster, indicating similarities between the Rip1Tag2 mouse model and human PanNET (Sadanandam et al. 2015).

Further, combination of miRNA and mRNA profiles revealed enrichment of distinct miRNA clusters in mRNA profiling subtypes of human PanNET and led to the definition of the three distinct groups, insulinoma-like tumours, intermediate tumours and metastasis-like primary tumours (Sadanandam et al. 2015). The comparative analysis of human and mouse PanNET using miRNA and mRNA profiles showed overlap of the subtypes, except intermediate tumours, which are mostly MEN1 or DAXX and ATRX deficient tumours, and for this reason, not represented by the Rip1Tag2 mouse model. The identification of these molecular groups may have application for selection of specific patient treatment and identification of new targets.

Small intestine NET

Two miRNA profiling studies have been conducted on 8 and 24 SI-NET, respectively (Ruebel et al. 2010, Li et al. 2013). In both, metastatic tumours were compared to primary tumours, in order to underline tumour progression. Several miRNAs were found to be deregulated and the results were validated by in situ hybridization and Northern blot analysis. Merging the outcomes from the two groups, we found in both groups downregulation of miR-133a and upregulation of miR-183 (metastases vs primary). miR-133a has two subtypes, including miR-133a-1 and miR-133a-2, which are located on chromosomes 18q11.2, 20q13.33 (Mitchelson & Qin 2015). Low expression of miR-133a is associated with poor prognosis and promotion of tumorgenesis in several cancers (Kawakami et al. 2012, Wu et al. 2012, Wang et al. 2014a,b, Lai et al. 2015, Mirghasemi et al. 2015). Several studies have confirmed that members of the miR-183/182/96 cluster are abnormally expressed in many tumours and are closely related to human cancers. Increased expression of this cluster was shown in glioma and breast cancers (Tang et al. 2013, Song et al. 2016). miR-183 was reported to promote migration and invasion in osteosarcoma (Zhu et al. 2012) and to be correlated with shorter overall survival in prostate cancer (Ueno et al. 2013).

Pulmonary carcinoids

Different studies have examined miRNA expression in pulmonary carcinoids using different profiling platforms comparing normal lung tissue and tumour, carcinoids and high-grade NEC as well as localized and metastatic disease (Lee et al. 2012, Deng et al. 2014, Mairinger et al. 2014, Rapa et al. 2015). Although these studies are difficult to merge, some recurrent deregulated miRNAs in lung NET progression were identified, including miR-129-5p, miR-409-3p and miR-155. miR-409-3p was found to be downmodulated in carcinoids with lymph node metastases (Rapa et al. 2015). It has been shown that miR-409-3p inhibit cell migration and/or invasion in fibrosarcoma (Weng et al. 2012), bladder cancer (Xu et al. 2013) and gastric cancer (Zheng et al. 2012) cells. In addition, miR-21 upregulation was correlated with metastatic disease (Lee et al. 2014, Rapa et al. 2015), which was also observed in PanNET (Roldo et al. 2006). This miR was shown to deregulate PTEN (Meng et al. 2007), involved in the mTOR pathway and ANP32A/SMARCA4 (Schramidei et al. 2011), two proteins that are involved in chromatin remodelling.

Conclusions

In conclusion, different mechanisms of tumorigenesis contribute to NET formation. This is particularly evident for PanNET for which most data are available, but likely also true for other NET entities.

Chromatin remodelling and telomere length maintenance, DNA damage and mTOR signalling are recurrent pathways involved in PanNET tumorigenesis (Scarpa et al. 2017). These mutation-driven mechanisms are crucial for PanNET development and malignant transformation, yet, are not sufficient to fully explain transcriptional groups and stratify patients regarding prognosis and treatment selection. Indeed, mutations in the recurrent genes MEN1, DAXX, ATRX and mTOR
pathway members seem equally distributed in the three PanNET groups based on RNA expression (Scarpa et al. 2017), suggesting that the reported driver mutations alone are insufficient to induce the transcriptome phenotype. Other mechanisms regulating gene expression on epigenetic levels could partially fill this gap. Intriguingly, despite the heterogeneity in gene expression, DAXX and ATRX mutations occur mainly in G2 tumours with poor prognosis, pointing out the relevance of these genes in stratifying patients and driving tumour evolution, accompanied by vast changes of DNA methylation (Pipinikas et al. 2015).

Activation of hypoxia signalling is a second phenotype (on RNA and protein level) specifically associated with aggressive PanNET (Couvelard et al. 2008, Schmitt et al. 2009). However, it still needs to be proven if and to what extent (epi)genetically induced pseudo-hypoxia signalling is driving tumour progression and eventually metastasis formation or it rather represents a consequence of tumour size and vessel architectures. Nevertheless, hypoxia signalling and low microvessel density remain important markers of tumour prognosis and a targetable mechanism of PanNET tumorigenesis (Couvelard et al. 2005). The lack of mutations in this pathway except for VHL in rare inherited patients, may implicate an epigenetic regulation. Indeed, VHL promoter methylation has been found in 6% of the tumours, suggesting that not only the lack of oxygen but also the epigenetic status could be responsible for hypoxia signalling (Schmitt et al. 2009).

Next to hypoxia, a stemness/progenitor cell-like phenotype is described in more malignant (MLP) PanNET, while beta cell differentiation marker expression is more prominent in the intermediate and insulin-like tumours, possibly suggesting a different cell of origin for the tumour subtype (Sadanandam et al. 2015). However, it is not solved whether this hypothesis holds true or there is more frequently a stepwise progression with a re-expression of stem-cell markers. It is well known in human PanNET that expression of the two stemness markers CK19 and c-Kit is associated with adverse outcome (Schmitt et al. 2007, Zhang et al. 2009).

Telomere biology is a further fundamental contributor to tumour progression and prognosis, and possibly a future therapeutic target. Telomere length maintenance via ALT activation in well-differentiated DAXX/ATRX mutated PanNET and TERT promoter mutations, rarely described in MEN1 and VHL patients, are consistent with a PanNET origin from not self-renewing cells, needing to re-lengthen the telomeres (Killela et al. 2013, Vinagre et al. 2016).

Finally, the high frequency of mutations in genes encoding for epigenetic modifiers suggest a fundamental role of epigenetics in progression of well-differentiated PanNET. Chromosomal instability is a main driver of NET tumorigenesis, which is highly associated with DNA methylation changes both in SI-NET and PanNET (Marinoni et al. 2014, Pipinikas et al. 2015, Karpathakis et al. 2016, Marinoni et al. 2017). In such a context, mutations in gene regulating chromatin status could induce changes in the epigenetic status leading to CIN. Both CIN and epigenetic changes contribute to the transcriptome dysregulation eventually defining the different biological and prognostic subtypes. Only big international cooperative studies will help defining these subtypes and the different pathways leading to them allowing better clinical exploitation of the knowledge.

We believe that the dissection of epigenetic changes and of the mechanisms regulating them will be crucial for the further molecular understanding of NET. Characterization of a specific epigenetic subtype of NET may lead to a better patient stratification than the current one, due to the high dependency of NET development on those changes and the increasing availability of specific epigenetic modifiers as therapeutic agents.

Declaration of interest

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

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