Molecular prognostic factors in small-intestinal neuroendocrine tumours

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Abstract

Background: Small-intestinal neuroendocrine tumours (SI-NETs) represent a heterogeneous group of rare tumours. In recent years, basic research in SI-NETs has attempted to unravel the molecular events underlying SI-NET tumorigenesis.

Aim: We aim to provide an overview of the current literature regarding prognostic and predictive molecular factors in patients with SI-NETs.

Method: A PubMed search was conducted on (epi)genetic prognostic factors in SI-NETs from 2000 until 2019.

Results: The search yielded 1522 articles of which 20 reviews and 35 original studies were selected for further evaluation. SI-NETs are mutationally quiet tumours with a different genetic make-up compared to pancreatic NETs. Loss of heterozygosity at chromosome 18 is the most frequent genomic aberration (44–100%) followed by mutations of CDKN1B in 8%. Prognostic analyses were performed in 16 studies, of which 8 found a significant (epi)genetic association for survival or progression. Loss of heterozygosity at chromosome 18, gains of chromosome 4, 5, 7, 14 and 20p, copy gain of the SRC gene and low expression of RASSF1A and P16 were associated with poorer survival. In comparison with genetic mutations, epigenetic alterations are significantly more common in SI-NETs and may represent more promising targets in the treatment of SI-NETs.

Conclusion: SI-NETs are mutationally silent tumours. No biomarkers have been identified yet that can easily be adopted into current clinical decision making. SI-NETs may represent a heterogeneous disease and larger international studies are warranted to translate molecular findings into precision oncology.

Introduction

Well-differentiated neuroendocrine tumours (NETs) represent a heterogeneous group of rare tumours, which have a relatively indolent disease course. Primary NETs can arise from neuroendocrine cells at various anatomic sites. They most commonly develop in the gastrointestinal tract and bronchopulmonary system (¹, ², ³). NETs can be classified as functional or non-functional, based on whether they cause clinical symptoms as a result of hormone secretion or not. In patients with metastatic small-intestinal NETs (SI-NETs), the carcinoid syndrome is common, which is characterised by diarrhoea, episodic flushing, bronchospasm and often carcinoid heart disease leading to right valvular dysfunction (⁴). Patients with non-functional SI-NETs are often asymptomatic or experience non-specific symptoms resulting in metastatic disease at the time of diagnosis in 27–73% of patients (¹, ², ³). In contrast to pancreatic NETs, SI-NETs are not known to arise in the context of hereditary syndromes,
for example multiple endocrine neoplasia (MEN) type 1 or 2 and Von Hippel Lindau disease.

The reported incidence of SI-NETs has increased over the last four decades, from 0.2 per 100,000 individuals in 1973 to 1.25 per 100,000 individuals in 2012 (5). This progressive rise can mainly be contributed to more frequent use and improvements of diagnostic modalities or alterations in pathological disease definition (2, 5). In the group of gastroenteropancreatic NETs, SI-NETs are second most prevalent after rectum NETs and followed by pancreatic NETs (5). Moreover, SI-NETs are the most frequent cancer type of the small intestine (6).

Currently, treatment for patients with SI-NETs is based on the availability of several treatment modalities, for example, surgery, liver-directed therapies, somatostatin receptor analogues and peptide receptor radionuclide therapy rather than on precision medicine. In case of non-functional, advanced and progressive SI-NETs, everolimus, targeting the PI3K/AKT/mTOR (mammalian target of rapamycin) pathway, has demonstrated anti-proliferative effects (7, 8, 9). However, there is no biomarker available that predicts response to everolimus.

To conclude, personalised treatment based on molecular profiling has not yet entered the arena of treatment modalities in advanced SI-NETs.

In order to move towards precision medicine, the genomic landscape of SI-NETs has been under increasing investigation over the past years in the hope of unravelling the molecular events underlying NET tumorigenesis, facilitating the identification of novel therapeutic targets, rational (targeted) therapy management strategies and to improve prognosis. Recently, whole-genome sequencing of primary pancreatic NETs revealed several genomic events which characterise their pathogenesis and are associated with tumour progression (10). In general, gene expression-based subtyping has led to new classifications of multiple tumour types. In contrast, the genomic landscape of SI-NETs remains poorly elucidated and biomarkers have not yet been identified. Moreover, the genetic constitution of SI-NETs has been shown to differ compared to pancreatic NETs (11). With this review we aim to provide the clinician treating SI-NETs with an overview of the recent studies evaluating molecular characteristics of SI-NETs and their predictive and prognostic significance.

Methods

A literature search was performed in PubMed in March 2019. As our main objective was to provide an up to date overview of the current literature regarding prognostic molecular factors in SI-NETs for clinicians treating patients with SI-NETs, we did not aim to perform a formal systematic review. The domain of this search consisted of adult patients with sporadic SI-NETs, the determinant of genetic or epigenetic alterations and the outcomes of prognosis, survival or progression. Synonyms of SI-NETs and (epi)genetic alterations with the outcome described as prognosis, survival and progression were used for the search. Search terms and syntax are described in detail in Table 1. Screening based on title and abstract was conducted by one reviewer, in case of uncertainties a second reviewer was consulted. Citation search of the included articles was performed to identify additional original studies.

Inclusion criteria consisted of patient populations >18 years, human, full-text available in English, published between 01/01/2000 and 01/03/2019 and studies on gastroenteropancreatic NETs. Studies with a patient population with underlying genetic syndromes, no separate genome analysis for SI-NETs, using previously published results and on the taxonomy of SI-NETs were excluded.

Results

The PubMed search yielded 1522 hits, of which 1461 articles were excluded after screening of title and abstract (Fig. 1). Following the full-text screening of 61 articles,
14 articles were excluded. The citation search identified 22 additional articles of which 7 were excluded. In total 55 relevant articles were found, consisting of 20 reviews and 35 original studies. The results of the selected original studies are shown in Table 2. Our review will discuss the most relevant studies, with a special focus on the prognostic implications of the identified molecular alterations. The identified studies describe different genomic events and altered expression of several proteins which play a key role in various molecular pathways involved in SI-NET tumorigenesis. Events which have been described in multiple studies and are discussed in this paper are shown in Fig. 2.

Genetics of SI-NETs

Chromosomal aberrations

From genomic profile studies, two different groups of SI-NETs can be identified, one which is characterised by loss of heterozygosity (LOH) of chromosome 18 as an early event and the other group which has no alterations of chromosome 18 and shows clustered gains on chromosomes 4, 5, 7, 14 and 20 (11, 12, 13, 14, 15).

Multiple studies reported loss of one copy of chromosome 18, with an incidence of 44–100% in primary SI-NETs (11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22). Chromosome 18 harbours several candidate tumour suppressor genes, including DCC (deleted in colorectal cancer; involved in axon guidance), SMAD4 (mothers against decapentaplegic homolog 4; TGFB signal transduction), SMAD2 (mothers against decapentaplegic homolog 2; TGFB signal transduction) and TCEB3C (transcriptional elongation factor B polypeptide 3C; encoding Elongin A3; RNA transcription). Banck et al., who performed whole-exome sequencing (WES) on 48 well-differentiated SI-NETs, found SMAD2 and SMAD4 monoallelic deletions in 21 tumours (22, 23).

Nieser et al. (n = 148) performed the first comprehensive study to identify chromosome 18 related events at genetic, epigenetic and gene/protein expression level, which only found DCC to be affected by the monoallelic loss of chromosome 18 (22). In addition, Simbolo et al. (n = 52) observed copy loss of multiple genes located on chromosome 18: CDH19 (cadherin 19; cell adhesion; 46.2%), BLC2 (B-cell-lymphoma; regulation of cell death; 42.3%), DCC (42.3%) and SMAD4 (28.8%) (15).
| Study            | Publication year | No. of patients | Domain                                           | Analysis technique                                                                 | Molecular aberrations                                      | Prognostic association | Remarks                          |
|------------------|------------------|-----------------|--------------------------------------------------|-----------------------------------------------------------------------------------|-------------------------------------------------------------|------------------------|----------------------------------|
| Löllgen et al.   | 2001             | 8               | Metastatic midgut NETs (6 ileal, 1 ileocecal valve, 1 ascending colon) | Genome-wide LOH* screening with microsatellite markers                            | Deletions on Chr 18 in 88% of midgut NETs                   | –                     | Analysis included 1 colon NET    |
| Wang et al.      | 2005             | 47              | Ileal NETs (n = 16)                               | Microsatellite markers, PCR amplification, sequencing of the BRAF gene            | Allelic loss of both arms of chromosome 18 in 69% No BRAF mutations were identified | –                     | –                                |
| Zhang et al.     | 2006             | 33              | SI-NETs with matched primary and metastatic tumours | Methylation-specific PCR, Western blot and immunochemistry                         | Methylation of RASSF1A and CTNNB1 promoters more frequent in metastatic vs primary tumours (P = 0.013 and P = 0.004, respectively) | –                     | –                                |
| Kim et al.       | 2007             | 29              | Well-differentiated ileal NETs (n = 15)           | Genome-wide high-density single-nucleotide polymorphism array analysis Pyrosequencing | Loss of Chr 18 in 67%, loss of Chr 21 or 21q in 13%         | –                     | –                                |
| Choi et al.      | 2007             | 35              | Ileal NETs (n = 15)                               | Genome-wide high-density single-nucleotide polymorphism array analysis Pyrosequencing | Hypomethylation of LINE-1 was greater in ileal NETs than in non-ileal and pNETs (P = 0.047), and tumours with lymph node metastasis (P = 0.02). Chr 18 loss (P = 0.001) and RAS-association domain family L isoform A gene methylation (P = 0.02). | –                     | –                                |
| Kulke et al.     | 2008             | 18              | Primary and metastatic SI-NETs (n = 24)           | High resolution arrays of single-nucleotide polymorphisms                          | Loss of Chr 18 in 61%, Chr 9 in 33% and Chr 16 in 22%. Gains on Chr 4 (33%) and Chr 5, 7, 20 and 14q (17%) | –                     | –                                |
| Andersson et al. | 2009             | 43              | Ileal NETs                                        | High-resolution array based on comparative genomic hybridisation                   | Loss of Chr 18 in 74%. Other frequent copy number alterations were gain of Chr 4 (30%), 5 (28%), 14 (23%) and 20 (33%), and loss of 11q22.1-q22.2, 11q22.3-q23.1 and 11q23.3 (21%) and loss of 16q12.2-q22.1 and 16q23.2-qter (16%) | –                     | –                                |
| Cunningham et al.| 2010             | 45 (37 sporadic and 8 familial)                  | Sporadic and familial ileal NETs (61 tumour samples)                                | High-resolution genomic and gene expression profiling                             | Chr 18 aberrations in both sporadic and familial ileal NETs (100 vs 38%) Frequent gain of Chr 7 in metastasis vs primary tumour (16 vs 0%) | –                     | –                                |
| Year | Authors | Sample Size | Study Design | Methodology | Findings |
|------|---------|-------------|--------------|-------------|----------|
| 2010 | Ruebel et al. | 8 | Primary and metastatic ileal NETs | RT-PCR, miRNA expression assay, Northern blotting in situ hybridisation | Downregulation of miRNA-133a (ratio 0.27*), −145 (ratio 0.33*), −146 (ratio 0.36*), −222 (ratio 0.41*) and −10b (ratio 0.44*) in 100% of primary vs matching metastasis, upregulation of miRNA-183 (ratio 1.39*) −48 (ratio 1.56*), and −19a+b (ratio 1.31*) in 75% metastatic ileal NETs compared to primary tumours |
| 2011 | Walsh et al. | 239 cases and 110 controls | Ileal NETs | Genome-wide association study single-nucleotide polymorphism genotyping | No single-nucleotide variants significantly associated with ileal NETs, rs2208059 in KIF16B had a trend towards statistical significance |
| 2011 | Edfeldt et al. | 19 SI-NETs (n = 18), lymph node metastases (n = 17), liver metastases (n = 7) | Gene expression arrays, qPCR | Three clusters of gene expression profiles were identified distinguishing primary tumours (11/18) from lymph node metastases (5/17) and a third group consisting of liver metastases (7/7), lymph node metastases (12/17) and primary tumours (7/8). The different profiles suggest changes in the development from primary tumour to metastases |
| 2012 | Stricker et al. | 58 SI-NETs (n = 17) | Pyrosequencing | LINE1 hypomethylation was detected in 82% of SI-NETs |
| 2013 | Banck et al. | 48 SI-NETs | Exome sequencing | 0.1 SNVs* per 108 nucleotides No recurrent mutations in cancer genes. 197 protein-altering SNVs affected multiple cancer genes, including FGFR2, MEN1, HOOK3, EZH2, MLF1, CARD11, VHL, NONO and SMAD1. Mutually exclusive amplification of AKT1 or AKT2 was the most common event in 16 patients with alteration of P13K/Akt/mTOR signalling |
| 2013 | Francis et al. | 180, including 48 from Banck et al. | SI-NETs | Frameshift mutations of CDKN1B in 8% SI-NETs (8%; 95% CI 4.7-12.7), hemizygous deletions encompassing CDKN1B in 14% |

*Mean metastatic/primary tumours ratio <1 (upregulated in primary tumours) *Mean metastatic/primary ratio >1 (downregulated in primary tumours)
Table 2

| Study          | Publication year | No. of patients | Domain | Analysis technique | Molecular aberrations | Prognostic association | Remarks |
|----------------|------------------|-----------------|--------|--------------------|-----------------------|------------------------|---------|
| Li et al.      | 2013             | 24              | SI-NETs (5 primary tumours, 5 mesentery metastasis, 5 liver metastasis) | Affymetrix Genechip miRNA array, qRT-PCR, Northern blot Analysis | miRNA-96 ($P < 0.01$ compared to mesentery metastasis (MM) and $P < 0.001$ liver metastases (LM)), $-182$ (MM $P < 0.05$, LM $P < 0.001$), $-183$ (MM $P < 0.001$, LM $P < 0.01$), $-196a$ (MM $P < 0.001$, LM $P < 0.001$) were upregulated during tumor progression |
|                |                  |                 |        |                    |                       |                        |         |
|                |                  |                 |        |                    | miRNA-31 (MM $P < 0.05$, LM $P < 0.05$), $-129-5p$ (MM $P < 0.01$, LM $P < 0.001$), $-133a$ (MM $P < 0.05$, LM $P < 0.001$) and $-215$ (MM $P < 0.05$, LM $P < 0.05$) were downregulated |
| Hashemi et al. | 2013             | 30              | SI-NETs ($n = 18$) and metastases ($n = 12$) | Comparative genome hybridisation, qPCR | Loss of chromosome 18 in 70%. Copy number losses on chromosome 11 (23%), 16 (20%), and 9 (20%), with regions of recurrent copy number loss identified in 11q23.1-qter, 16q12.2-qter, 9pter-p13.2 and 9p13.1-11.2. Gains detected in chromosomes 14 (43%), 20 (37%), 4 (27%), and 5 (23%) with recurrent regions of copy number gain in 14q11.2, 14q32.2-32.31, 20pter-p11.21, 20q11.1-11.21, 20q12-qter, 4 and 5. Differences between primary tumours and metastases; loss of 16q ($P = 0.003$) loss and gain of Chr 7 ($P = 0.016$). Gain in 20pter-p11.21 was associated with short survival ($P = 0.013$). No other significant associations were observed between recurrent copy number alterations and survival |
| Bottarelli et al. | 2013         | 30              | Ileal NETs | DNA fragment analysis and sequencing of the mutation cluster region of the APC gene | APC gene mutations in 23%, of which missense (57%) and nonsense/frameshift (14%) mutations |
| Edfeldt et al. | 2013             | 43              | SI-NETs | Gene copy number determination by PCR, real time quantitative RT-PCR, RNA interference, CpG methylation pyrosequencing | One copy deletion in 89% SI-NETs with reduced Elongin A3 expression in 77%. No association was found with tumour progression |
| Fotouhi et al. | 2014 | 33 | SI-NETs (n = 44) | Pyrosequencing, ELISA-based quantification of global DNA methylation, qRT-PCR |
|---------------|------|----|----------------|--------------------------------------------------------------------------------|
|               |      |    |                | Methylation was seen in WIF1 (methylation index (MI) 50%, (16–92%), RASSF1A (MI 16% (1-49%), CTNNB1 (MI 13% (4-34%), CXCL14 (MI 14% (3-39%), NKK2-2 (MI 10% (2-28%), P16 (CDKN2A) (MI 4% (1-33%), LAMA1 (MI 10% (4-24%), and CDH1 (MI 8% (3-22%), APC (MI 3% (2-8%), CDH3 (MI 6% (3-12%), HIC1 (MI 5% (1-12%), P14 (CDKN2A) (MI 5% (2-17%), SMAD2 (MI 4% (1-8%), and SMAD4 (MI 3% (1-6%) had low levels of methylation. WIF1 methylation was significantly increased (P = 0.001) and WIF1 expression was reduced in SI-NETs vs normal references (P = 0.003). WIF1, NKK2-3 and CXCL14 expression was reduced in metastases vs primary tumours (P < 0.02). Global methylation of LINE1 was reduced in tumours vs normal references (65 vs 75%), and was associated with loss of Chr18p and 18q (P = 0.02, P = 0.003, respectively). |

| Verdugo et al. | 2014 | 20 | Matched primary SI-NETs (n = 10) and their mesenteric lymph node metastases (n = 10) | Human methylation 27 BeadChip array profiling |
|----------------|------|----|-----------------------------------------------------------------|---------------------------------------------|
|                |      |    | RUNX3, TP73 and CHFR were highly methylated (β value > 0.9). At Chr 18q21-pter (β value >0.7), SETBP1, ELAC1, MBDA1, MAPK4, and TCEB3C were methylated including several members of the Serpin peptidase inhibitor family (SERPINB3, SERPINB5). |

| Norlen et al. | 2014 | 15 | Percutaneous carcinomatosis of SI-NETs (n = 8) and controls (n = 7) | Single-nucleotide polymorphism array |
|---------------|------|----|--------------------------------------------------------------------------------|----------------------------------|
|                |      |    | Two groups were identified, group A with a greater proportion of patients with PC (86%) than group B (25%), with LOH of the entire or major part of chromosome 18 in group A (75%) compared to limited LOH (75%) or no LOH (25%) in group B |

| Crona et al. | 2015 | 200 | SI-NETS (n = 362) | Automated Sanger sequencing of the CDKN1B gene, immunohistochemistry |
|--------------|------|-----|-----------------|---------------------------------------------------------------------|
|              |      |     | CDKN1B mutations were present (33 and 11% respectively). Expression of p27 did not correlate with CDKN1B mutation status. No differences in clinical characteristics between CDKN1B mutated and CDKN1B wild-type tumour carriers were found |

Low expression of RASSF1A and P16 were associated with poor survival (P = 0.045 and P = 0.011, respectively). Gene-specific promoter methylation or global methylation did not influence survival.
Table 2 Continued

| Study                  | Publication year | No. of patients | Domain         | Analysis technique                                                                 | Molecular aberrations                                                                 | Prognostic association | Remarks                       |
|------------------------|------------------|-----------------|----------------|------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|------------------------|-------------------------------|
| Maxwell et al.         | 2015             | 90              | SI-NETs        | Exome sequencing and CNV analysis by quantitative PCR                               | CDKN1B frameshift mutations in 3.5% of SI-NETs (95% CI: 1.1–9.8%), 1 patient had a hemizygous deletion of CDKN1B and 2 patients duplications (3.4%; 95% CI 0.41–7.2%), Mutations of CDKN1B occurred in 6.9% | –                      | –                             |
| Delgado Verdugo et al. | 2015             | 7               | SI-NETs        | Whole-exome capture, NGS*, high resolution SNP array, copy number variation analysis | Loss of Chr18 in 71% of SI-NETs. No tumour-specific somatic mutation was identified       | –                      | –                             |
| Bollard et al.         | 2015             | 38              | Ileal NETs     | Immunochemistry, methylation-specific PCR                                           | SEMA3F expression was lost in 96% ileal NETs and all their metastases. SEMA3F loss of expression was associated with promoter gene methylation (no P value provided) | –                      | –                             |
| Karpathakis et al.     | 2015             | 97              | SI-NETs        | Whole-genome or targeted CDKN1B sequencing, Human methylation 450 BeadChip array profiling, methylated DNA immunoprecipitation sequencing, CNV analysis, whole-genome DASL* expression array profiling | Subgroup 1: chromosome 18 LOH, CDKN1B mutations, CIMP*, negativity. Subgroup 2: absence of arm-level CNVs, CIMP positivity Subgroup 3: multiple CNVs Epimutations were found at a recurrence rate up to 85%, and 21 epigenetically dysregulated genes were identified, including CDX1 (86%), CELSR (84%), FBP (84%), and GPR (84%). | 3 subgroups of SI-NETs with different PFS* (not reached at 10 years vs 56 months vs 21 months; P = 0.04) | –                             |
| Miller et al.          | 2016             | 90              | Primary SI-NETs (n = 28), adjacent normal small bowel (n = 14), matched lymph node metastases (n = 24), normal lymph node metastases (n = 7), normal liver (n = 2) and liver metastasis (n = 15) | NanoString miRNA profiling, qRT-PCR, luciferase reporter assays and immunoblotting        | miR-204-5p (P = 2.44 × 10^-67), miR-7-5p (P = 2.57 × 10^-144) and miR-375 (P = 6.30 × 10^-4) were upregulated and miR-1 (P = 0.0004) and MiR-143-3 (P = 8.11 × 10^-3) were downregulated in lymph node and liver metastases vs primary tumours | –                      | –                             |
| Andersson et al.       | 2016             | 33              | Well-differentiated distal ileal NETs | Genome-wide sequencing                                                            | Loss of chromosome 18 in 65% and gains of chromosome 4,5,7,14 and 20 in 51%. Loss of CDKN1B in 8%. 3 subgroups were identified. The prostaglandin E receptor 2 (PTGER2) is the most activated in tumours of higher grade (P = 4.4 × 10^-18), whereas Forkhead box M1 (FOXM1) was the most activated regulator in tumours with gain of chromosome 14 (P = 2.5 × 10^-4) | The largest subgroup (n = 17) was characterised by longer survival (P<0.05) and higher expression of neuroendocrine markers, including SSTR2. Tumours with higher grade (G2/3) or gain of chromosome 14 were associated with shorter patient survival (P<0.05) and increased expression of cell cycle-promoting genes | Analysis included 1 ileal NEC | –                             |
| Authors          | Year | Subjects | Methods                                                                 | Findings                                                                                                                                                                                                 | Notes     |
|------------------|------|----------|--------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------|
| Dumanski et al. | 2017 | 239      | Sporadic (215) and familial (24) SI-NETs compared to three control cohorts with 35,688 subjects | A mutation in the MUTYH gene was significantly enriched in SI-NETs (both sporadic and familial) compared to controls (OR 5.09; 95% CI 1.56–14.74; P = 0.0038)                                                              | -        |
| Karpathakis et al.| 2017 | 20       | SI-NETS and matched liver metastasis                                      | Human methylation 450 BeadChip array profiling, methylated DNA immunoprecipitation sequencing, whole-genome DASL* expression array profiling                                                               | -        |
| Shi et al.       | 2017 | 267      | SI-NETs (n = 55)                                                         | CDKN1B mutations in 10.9%. No clear association was found between CDKN1B mutation and protein expression                                                                                             | -        |
| Nieser et al.    | 2017 | 148      | qRT-PCR, Western blot, immunohistochemistry, NGS*, SNP array analysis, miRNA analysis by qRT-PCR | Chr 18 LOH in 65%. Only DCC (deleted in colorectal cancer) revealed loss of/greatly reduced expression in 29%. No additional genetic or epigenetic alterations were present on Chr18. Loss of GABLES did not correlate with survival. | -        |
| Keck et al.      | 2018 | 12       | Matched small bowel tissue, primary SI-NETs, liver metastases            | Serial differential expression was validated in 7/10 genes, with several interacting members of the AKT, MYC, or MAPK3 pathways. Liver metastases had underexpression of PMP22 (P < 0.001) High expression of SERPINA10 (primary p < 0.001, liver metastases <0.001) and SYT13 (primary P < 0.001, liver metastases <0.001) was characteristic of primary SI-NETs and liver metastases | -        |
The clinical significance of LOH of chromosome 18 has been evaluated in multiple studies, either focusing solely on LOH of chromosome 18 or as part of a molecular profile study. According to Andersson et al. (n = 43) LOH of chromosome 18 is associated with worse overall survival (13). In contrast, Kim et al. did not find a significant correlation between loss of chromosome 18 and survival (18). Contrarily, Yao et al. (n = 89) found that SI-NETS with low generalised chromosomal instability (CIN) (which consisted of a cluster with LOH of chromosome 18) displayed significantly longer median PFS than those with a high CIN (which consisted of 3 clusters with different combinations of gains of chromosome 4, 5, 7, 14, 20 and 1 cluster with copy number gains across most chromosomes). PFS in patients with a low CIN (n = 55) was 18.6 vs 9.2 months in high CIN (n = 38) (HR; 0.41; 95% CI 0.24–0.73; P = 0.0021) (11).

As described by the clusters of Yao et al., recurrent gains of chromosome 4, 5, 7, 14 and 20 are common in SI-NETs (11, 12, 13, 15, 18, 19, 21). In two studies by Andersson et al., gain of chromosome 14 was seen in 6 of 32 well-differentiated SI-NETs and was associated with higher tumour grade and shorter survival (HR 8.39; 95% CI 3.04–23.11) (13, 21). However, Cunningham et al. (n = 45) and Simbolo et al. (n = 52) could not corroborate these findings (15, 19). Hashemi et al. (n = 30) studied copy number alterations (CNAs; gains and losses of areas of the genome, immunohistochemistry and losses on chromosomes 11 and 18) (P = 0.0047) (315). Frequent allelic loss of 4 genes located on Chr 18 (BCL2, CDH19, DCC and SMAD4) in 44.2% and losses on chromosomes 11 and 16 (38%) and 15%. Gains on chromosomes 4 (31%), 5 (27%), 14 (36%), and 20 (20%).

Mutational status

Banck et al. analysed 48 primary SI-NETs, predominantly grade 1, by massively parallel exome sequencing and detected a low mutation rate in the SI-NET genomes with an average of 0.1 somatic single-nucleotide variants (SNVs) per 10⁶ nucleotides in the exome, suggesting that SI-NETs are mutationally quiet tumours (23). No recurrent mutations in the 215 sequenced target genes were found. In the studied SI-NETs, 197 protein-altering SNVs were detected a low mutation rate in the SI-NET genomes (SNVs) per 10⁶ with an average of 0.1 somatic single-nucleotide variants (SNVs) per 10⁶ nucleotides in the exome, suggesting that SI-NETs are mutationally quiet tumours (23). No recurrent mutations in the 215 sequenced target genes were found. In the studied SI-NETs, 197 protein-altering SNVs were
common. Amplifications were also observed at the PDFFDR (platelet-derived growth factor receptor alpha) locus in 20.8% (23). In a recent study by Simbolo et al. frequent copy gains were detected in AKT1 (30.8%) and PDGFRα (platelet-derived growth factor receptor alpha: 28.8%) as well. Furthermore, gains were present at the FOS gene (transcription factor subunit; 36.5%), KIT (involved in cell proliferation, survival, migration and differentiation; 28.8%) and KDR (kinase insert domain receptor, involved in VEGF signalling; 28.8%) genes (15). Higher mutation rates in primary SI-NETs were associated with increased likelihood of recurrent liver metastases (P<0.04) (23). In a study by Francis et al. (n=180) including 48 cases from Banck et al., heterozygous frame shift mutations of the cyclin-dependent kinase inhibitor 1B gene (CDKN1B) in 14 of 180 SI-NETs (8%; 95% CI 4.7–12.7%) were observed (24). CDKN1B is located on chromosome 12 and encodes the protein p27kip1, a cyclin-dependent kinase inhibitor (CKI), whose main function is to control the progression from G1 to S phase in the cell cycle. The reported mutations in this putative tumour suppressor gene in SI-NETs are loss-of-function truncating mutations throughout the gene; no hotspot has been identified.

A further study by Crona (n=200), confirmed the presence of CDKN1B mutations in 17 of 200 SI-NETs (8.5%) (95% CI 4.6–12.4%) (26). Mutational status did not appear to correlate with protein expression of p27kip1 and no immediate detectable impact on clinical phenotype and survival was found (26). Similarly, Shi et al. observed CDKN1B mutations in 10.9% of 55 SI-NETs and found no association between CDKN1B mutation, p27kip1 expression and survival (27). Only a trend towards shorter survival of patients with tumours exhibiting low expression of p27kip1 (multivariate HR, 2.04; 95% CI 1.06–3.93; P=0.03) was observed. Other studies found CDKN1B mutations in 4.5–9.6% of SI-NETs (11, 15, 28, 29). Furthermore, Yao et al., using whole-exome and targeted panel sequencing on 89 SI-NETs from the RADIANT trials, found recurring mutations in BCOR (BCL-6-interacting corepressor) in 5.6% (11). BCOR has interactions with histone deacetylases which are involved in the regulation of gene expression through DNA methylation (11). Another recent study, by Simbolo et al., performed targeted sequencing on 52 primary SI-NETs of which 34.6% showed somatic mutations (15). APC (adenomatous polyposis coli; Wnt signalling pathway regulator) and CDKN2C

Figure 2
The studies presented in this review identified the deregulation of the expression of multiple genes in SI-NETs, which are commonly associated with carcinogenesis in other tumours. In the figure above, only those molecular alterations that have been found in multiple studies are depicted, together with their presumed role as key regulators of different cell functions and their possible effect on tumour progression as defined by the hallmarks of cancer (55).
Molecular alterations in primary tumours vs metastases

Molecular differences between primary tumours vs metastases can provide insight in the process of tumour progression. Cunningham et al. observed increased gains of chromosome 7 in metastases (30 mesenterial and 4 hepatic) vs primary SI-NETs (16 vs 0%) (19). Correspondingly, Hashemi et al. reported frequent gain of 7q22.3-qter in metastases (12 regional and 7 distant; \( P=0.016 \)) compared to primary tumours (14). Loss of 16q12.2qter was more common in distant metastases vs primary tumours (\( P=0.003 \)) (14). Karpathakis found LOH of chromosome 18 in 79% in liver metastases (31). In the same study, amplification of chromosome 20 was found in 42%, deletion of chromosome 19 in 34% and gain of chromosome 17q in 21% of liver metastases (31). Furthermore, at mRNA level, analysis of differentially expressed genes between liver metastases and primary tumours identified significant enrichment of multiple cancer-related pathways overexpressed in liver metastasis, for example P13K signalling events, ERBB1 downstream signalling, PDGFRB signalling and the mTOR pathway (adjusted \( P<0.001 \)) (31). Keck et al. demonstrated by RNA sequencing that liver metastases show underexpression of PMP22 (peripheral myelin protein 22; integral membrane protein involved in demyelinating disease and apoptosis) compared to the corresponding primary tumour (\( P<0.001 \)) (32). Fotouhi et al. (\( n=33 \)) found different expression levels of CXCL14 (chemokine CXC motif ligand 14; involved in cytokine activity and angiogenesis) mRNA in metastases compared to primary tumours (\( P=0.0016 \)), which correlates with methylation status of the respective genes (33). Furthermore, increased expression was found for mRNA encoding beta-catenin (involved in Wnt signalling pathway) in metastases compared with primary tumours (\( P=0.041 \)); for mRNA encoding P16 (regulates entry into S phase) in distant metastasis compared to primary tumours and regional metastases (\( P=0.015 \)) and for mRNA encoding RASSF1A (involved in cell cycle regulation, apoptosis and migration), in regional metastases compared to primary tumours and distant metastases (\( P=0.008 \)). Low mRNA expression of RASSF1A and P16 were each associated with short survival (\( P=0.045 \) and \( P=0.011 \), respectively) (33). Using gene expression arrays, Edfeldt et al. were able to identify differentially expressed mRNA in SI-NET metastases compared to primary tumours which resulted in the identification of three different gene expression clusters. However, these clusters did not correlate with tumour progression (34). To conclude, dissemination of SI-NETs is associated with genomic events; yet the way in which these events contribute to tumour progression remains unclear.

Prognostic stratification based on LOH of chromosome 18, CDKN1B mutations, CpG island methylator phenotype and copy number variations

Karpathakis et al. identified different prognostic subgroups using hierarchical clustering. In a sophisticated large-scale integrated genomic analysis, including DNA methylation, gene expression and copy number variance (CNV) of 97 SI-NETs from a cohort of 85 patients they identified three molecular subtypes of SI-NETs using an integrated genome analysis (29). The largest subgroup (55%) was defined by chromosome 18 LOH and is associated with the presence of CDKN1B mutations, and CpG island methylator phenotype (CIMP) negativity. The CpG island methylator phenotype refers to the DNA hypermethylation of promoter-associated CpG islands of tumour suppressor and DNA repair genes, which leads to transcriptional silencing of these genes. These patients had the most favourable PFS (not reached at 10-year follow-up) after resection and a median age of 67 years at diagnosis. A second subgroup (18%) was characterised by the absence of arm-level CNVs (copy number variations that span the chromosomal arm) and a high degree of CIMP positivity. This group had an intermediate PFS (56 months) and a younger median age at diagnosis (60 years). The third subgroup consisted of

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26% of SI-NETs and was characterised by multiple CNVs; these patients had a significantly poorer PFS (21 months) and were youngest at onset (54 years), suggesting a more aggressive clinical phenotype. In accordance with Karpathakis et al., Yao et al. (n=89), identified similar prognostic groups regarding LOH of chromosome 18 and alterations in chromosome 4, 5 and 20 (11). However, Simbolo et al. classified their cohort (n=52) into the three molecular groups of Karpathakis et al. and did not observe any statistically significant correlation with prognosis (P=0.73) (15). These results of Simbolo et al. could be due to the relatively small cohort in comparison to the cohort of Karpathakis et al. and do not necessarily weaken the findings of Karpathakis and Yao et al. Based on the findings of Karpathakis et al. and considering the relatively low frequency of somatic mutations in SI-NETs, it seems unlikely that mutations of the CDKN1B gene or LOH of chromosome 18 alone are driving the SI-NET tumorigenesis and suggests a greater role for epigenetic dysregulation (11, 15, 20, 25, 29, 35).

Germline mutations in SI-NETs

A germline mutation is defined as a mutation which occurs in reproductive cells and therefore is incorporated in every cell of the offspring. A study by Dumanski et al. (n=239) sequenced germline DNA from 24 patients from 15 families with a history of SI-NETs and from 215 sporadic SI-NET patients (36). A mono-allelic mutation causing an amino-acid substitution p. (Gly396Asp) in MUTYH was found to be significantly enriched in both patients affected with familial SI-NETs and in sporadic SI-NETs, compared to controls (minor allele frequencies 0.013 and 0.03, respectively) with an odds ratio of 5.09 (95% CI 1.56–14.74; P=0.0038). MUTYH encodes a DNA glycosylase, involved in repair of oxidative DNA damage in order to prevent mutation accumulation leading to tumorigenesis. Biallelic germline MUTYH mutations lead to multiple colorectal adenomas and carcinomas, referred to as MUTYH-associated polyposis (MAP), a recessive hereditary colorectal polyposis syndrome. Interestingly, MUTYH germline mutations were also found in pancreatic NETs (10, 37). By defective DNA repair, carriers with MUTYH mutations thus seem to have a predisposition to develop NETs of the pancreas or small intestine.

Epigenetics in SI-NETs

Epigenetic modification can be defined as a change in gene expression without alterations to the gene's DNA sequence itself (38). Since SI-NETs appear to have relatively few somatic mutations, epigenetic dysregulation could play an important role in the tumorigenesis of SI-NETs and may have important clinical implications (23, 24, 35). Epigenetic changes include DNA methylation, histone modifications and the actions of miRNA. Hypermethylation and hypomethylation and histone modifications modify gene expression, whereas miRNAs, small single-stranded RNA molecules, regulate gene expression post-transcriptionally.

These processes can be pharmacologically modified by targeting enzymes involved in DNA methylation and histone modifications, and by miRNA inhibitors, thereby representing an appealing target for therapy (35, 39).

In comparison with genetic mutations, epigenetic alterations are significantly more common and recurrent in SI-NETs. Our search yielded studies ranging from 8 to 97 patients that showed epigenetic alterations in SI-NETs for example DNA methylation changes in 65–82% of SI-NETs and multiple miRNA deregulations (29, 40, 41, 42). Several studies reported differences in methylation and miRNA patterns between primary tumours and metastases, suggesting a possible role in tumour development or progression. A recurrent event is the epigenetic silencing of RASSFIA (RAS-association domain family 1, isoform A gene; tumour suppressor gene inducing cell cycle arrest) expression by hypermethylation of its promotor. This event was observed by Choi et al., Zhang et al. and Fotouhi et al. and was more prominent in metastases than in primary tumours (33, 43, 44). In addition, increased hypermethylation of the CTNNB1 promoter was observed in liver metastasis compared to the corresponding primary tumours (44). Promotor gene methylation was also found in a study by Bollard et al. (n=38) in 96% of ileal NETs and their metastases. The expression of the axon guidance molecule SEMA3F (semaphorin 3F) was lost due to hypermethylation (45). SEMA3F expression is a negative regulator of MAPK and mTOR signalling pathways.

The first genome-wide DNA methylation analysis of SI-NETs performed by Verdugo et al. in 10 SI-NETs and ten matched mesenteric lymph node metastasis observed a high level of methylation in another gene set located at chromosome 18q21-qter (46). In these patients, high methylation index correlated with more malignant behaviour.

Karpathakis et al. found hypermethylation of the promoter region of the gastric inhibitory polypeptide receptor (GIPR; inhibits gastric secretion and gastrin release and stimulates insulin release) gene body in 74% of primary SI-NETs. Of note, in this study DNA
methylolation in SI-NETs was compared to the methylolation status of normal intestinal mucosa which normally expresses GIPR, whereas the methylolation status of enterochromaffin cells in the small intestine is unknown. Progressive hypermethylolation of this gene was seen in liver metastases compared to primary tumours (29, 31).

**MicroRNAs in primary tumours vs. metastases**

Two miRNA profiling studies (n=8, n=24, respectively) comparing primary SI-NETs to its respective metastases found multiple miRNAs to be deregulated during tumour progression (40, 47). A downregulation of miRNA-133a and upregulation of miR-183 was consistently found in metastases vs primaries. A study by Miller et al. (n=28) confirmed downregulation of miRNA133a and found differential expression of several other miRNAs in SI-NETs and their metastases (48).

**Discussion**

SI-NETs are rare tumours with a relatively indolent course. Unfortunately, treatment options are limited with minimal survival benefit. Therapies targeting somatostatin receptors, expressed by the majority of SI-NETs, are only able to stagnate disease progression temporarily. In an attempt to identify prognostic factors and new effective targets for precision medicine, the genomic landscape of SI-NETs has been under increasing investigation in recent years. LOH at chromosome 18 remains the most frequent genomic aberration (44–100%) found in SI-NETs (11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22).

The tumour suppressor gene, CDKN1B, is mutated in approximately 8% SI-NET patients (11, 15, 26, 27, 28, 29). Interestingly, CDKN1B is regulated by menin, the protein that is defect in the majority of patients with the inheritable MEN 1 syndrome (75–80%). Moreover, in MEN1 patients without mutations in the gene encoding menin (20–25%), CDKN1B was shown to be inactivated in some individuals (3.6%) (49). Thus, several SI-NETs and MEN1-associated endocrine tumours may share a common oncogenic pathway. Genetic alterations in the PI3K/AKT/mTOR were found in primary SI-NETs and liver metastasis, providing a rationale for the use of mTOR inhibitors (15, 23, 31, 45). However, a correlation between efficacy and mTOR mutational status prior to commencement of therapy with mTOR inhibitors has not yet been established. Daskalakis et al. (n=27) recently tested the *ex vivo* activity of several targeted kinase inhibitors and found great variability in *ex vivo* sensitivity for most drugs, emphasising the need for predictive biomarkers which could support clinical decision making (50).

Furthermore, mutations in APC, CDKN2C (both 7.7%) and BRAF, KRAS, PIK3CA and TP53 (each 3.8%) were recently identified in SI-NETs (15).

An association of (epi)genetic aberrations with prognosis was found in 16 of the 35 original studies reviewed. Karpathakis et al. (n=97) and Yao et al. (n=89) identified molecular subtypes of SI-NETs with significant difference in PFS (11, 31). However, validation of these subgroups in an independent and larger cohort is required before translation into clinical practice is possible. A gain of chromosome 14 and 20pter-p11.21 was associated with shorter survival in two studies (P<0.001, P<0.013 respectively) (13, 14). SRC copy number gains were associated with poorer prognosis (P=0.047) (11). Epigenetic alterations such as specific promoter methylation and global methylation and their effect on prognosis are yet to be determined (33, 41, 43, 44, 45, 46).

At present, predictive or prognostic biomarkers, which can be adopted into clinical practice, have not yet been established. Inactivated tumour suppressor genes, which are found in SI-NETs, are generally unsuitable as targets since restoring the function of tumour suppressor genes is difficult to accomplish. Mutations in oncogenes, which should be easier to target, have only recently been described in small numbers in SI-NETs and thus far no clinical studies have been undertaken to target these mutations in SI-NETs. Of note, Alvarez et al. identified the HDAC class inhibitor Etinostat as potent inhibitor of master regulatory activity for 42% of metastatic gastroenteropancreatic NETs, leading to the initiation of a clinical trial (NCT03211988) (9).

The low mutational burden found in SI-NETs may render these tumours less eligible for immunotherapy using immune checkpoint inhibitors because tumour mutational burden is an important determinant of clinical benefit to immune checkpoint blockade in most tumours. Additionally, the recently characterised tumour microenvironment in NETs, for example low expression for PD1 and PDL1 in SI-NETs, combined with a modest T-cell infiltrate, further tempers expectations regarding a response to the currently used PD1 and PDL-1 inhibitors, although this remains to be investigated (51).

More promising targets in SI-NETs may constitute the DNA methylation machinery. In comparison with genetic mutations, epigenetic alterations are significantly more common in SI-NETs. Specific genes such as RASSF1A, SEMA3F and CTNNB1 are hypermethylated in SI-NETs silencing their transcription (3, 43, 44, 45, 46).
RASSF1A hypermethylation is also observed in pancreatic NETs, lung NETs and thymic NETs, whilst it is not found in appendiceal NETs (52). During the last decades, an increasing number of drugs targeting DNA methylation and histone methylation have been developed and successfully tested pre-clinically which are currently in evaluation in phase I-III clinical trials (53). Additionally, the more specific upregulation of miRNAs in SI-NETS as described above may provide actionable targets since multiple strategies for miRNA-based therapies are under investigation (54).

In this era of accumulating studies regarding the molecular background of SI-NETS, we felt there was an unmet need to provide the clinician with an overview of (epi)genetic alterations and explain their relevance in terms of prognosis and possible novel therapeutic options. Despite our efforts to perform an extensive and broad search, studies may have been missed due to its non-systemic character. Limitations of studies used in this review include relatively small and heterogeneous cohorts, different genomic analysis techniques and paucity of relation of (epi)genetic aberrations to clinical outcomes.

The rarity of SI-NETs has hampered conducting sizeable clinical trials involving large-scale integrated genomic analysis. In the coming years, hopefully international collaborations will enable larger studies to be performed which correlate (epi)genetic alterations to clinical outcomes and aim to identify targetable (epi)genetic alterations. Larger studies combined with evolving molecular technologies might lead to a more effective treatment strategy in which patients with specific molecular tumour profiles will be selected for targeted pharmaceutical interventions.

Conclusion

SI-NETs have a low mutational burden, whereas epigenetic alterations are more prevalent. Mutations as described in pancreatic NETs are generally not observed in SI-NETS. Several studies identified (epi)genetic subtypes and molecular profiles of SI-NETS with significant difference in progression-free survival (PFS) and overall survival (OS). More research should be conducted to identify prognostic and predictive biomarkers that can be adopted in clinical decision making.

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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