### Prognostic Impact of Novel Molecular Subtypes of Small Intestinal Neuroendocrine Tumor

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**Abstract**

**Purpose:** Small intestinal neuroendocrine tumors (SINET) are the commonest malignancy of the small intestine; however, underlying pathogenic mechanisms remain poorly characterized. Whole-genome and -exome sequencing has demonstrated that SINETs are mutationally quiet, with the most frequent known mutation in the cyclin-dependent kinase inhibitor 1B gene (CDKN1B) occurring in only ~8% of tumors, suggesting that alternative mechanisms may drive tumorigenesis. The aim of this study is to perform genome-wide molecular profiling of SINETs in order to identify pathogenic drivers based on molecular profiling. This study represents the largest unbiased integrated genomic, epigenetic, and transcriptional analysis undertaken in this tumor system.

**Experimental Design:** Here, we present data from integrated molecular analysis of SINETs (n = 97), including whole-exome or targeted CDKN1B sequencing (n = 29). HumanMethylation450 BeadChip (Illumina) array profiling (n = 69), methylated DNA immunoprecipitation sequencing (n = 16), copy-number variance analysis (n = 47), and Whole-Genome DASL (Illumina) expression array profiling (n = 43).

**Results:** Based on molecular profiling, SINETs can be classified into three groups, which demonstrate significantly different progression-free survival after resection of primary tumor (not reached at 10 years vs. 56 months vs. 21 months, P = 0.04). Epimutations were found at a recurrence rate of up to 85%, and 21 epigenetically dysregulated genes were identified, including CDX1 (86%), CELSR3 (84%), FBPI (84%), and GIPR (74%).

**Conclusions:** This is the first comprehensive integrated molecular analysis of SINETs. We have demonstrated that these tumors are highly epigenetically dysregulated. Furthermore, we have identified novel molecular subtypes with significant impact on progression-free survival. *Clin Cancer Res; 22(1): 250–8. ©2015 AACR.*

### Introduction

The incidence of small intestinal neuroendocrine tumors (SINET) is increasing (1) and accounts for 25% of all NETs. To date, the underlying pathogenic mechanisms have been poorly characterized, and due to relatively rarity, these tumors have not been subject to large-scale integrated genomic analyses, such as The Cancer Genome Atlas (TCGA) or International Cancer Genome Consortium (ICGC) projects. The most frequent genomic event identified in SINETs is loss of heterozygosity (LOH) at chromosome 18, occurring in 60% to 78% of tumors (2, 3); more recently, recurrent mutations in the cell cycle regulator CDKN1B (cyclin-dependent kinase inhibitor 1B) have been identified in approximately 8% of tumors in large-scale sequencing studies (3, 4). Mutations occurring in CDKN1B are loss-of-function truncating mutations that occur throughout the gene. Mutational status does not appear to correlate with expression of p27 (the protein product of CDKN1B) and has no detectable association with clinical characteristics or survival (4). Given the low frequency of mutations in this putative tumor suppressor gene, and the absence of other obvious pathogenetic genome alterations, we hypothesized that alternative mechanisms such as epigenetic dysregulation are likely to play a role in these tumors (5).

Epigenetic alterations lead to changes in gene expression without modification of the underlying DNA sequence. DNA methylation and chromatin modifications are two of the mechanisms which regulate gene expression and are frequently disrupted in cancer. It has long been recognized that hypermethylation at the promoter region of a gene can lead to reduced gene expression (6). More recently, it has been demonstrated that hypermethylation over the gene body leads to increased gene expression, and demethylating the gene body by application of 5-aza-2-deoxycytidine leads to downregulation (7).

Epigenetic dysregulation is known to play a key role in other NET development, including tumors of pancreatic and bronchial origin (8, 9). Integrated molecular profiling based on methylation analysis has recently been shown to identify clinically distinct
subgroups of lung carcinomas, including a neuroendocrine-specific "epitype" (10).

Here, we present the first comprehensive integrated genome-wide molecular analysis of a large cohort of SINETs.

Materials and Methods

Patient recruitment

All patients provided written informed consent for their tissue to be analyzed in this study, which was approved by NHS Camden and Islington Community Research Ethics Committee (Ref: 09/H0722/27). A total of 97 tumors and 25 normal tissue samples were analyzed from 85 individuals. Inclusion criteria were histopathologic diagnosis of SINET, and surgical resection of either primary tumor or liver metastasis. Exclusion criteria were biopsy sampling of tumor only (due to inadequate amounts of DNA which was obtained from biopsy samples). Subjects were included from three international sites (UK, the United States, and Canada), and recruitment was performed during the period of 2011 to 2013. Anonymized clinical information was retrospectively collected from patient records where available including histopathologic details (tumor grade and stage) and progression-free survival (PFS) following resection. PFS was used as the primary endpoint for prognosis.

Pathology review

All cases were reviewed by two expert NET pathologists (T.V. Luong, M. Novelli, S.L. Asa, or S. Serra). The European NET Society grading system was used, which utilizes the Ki-67 proliferation index. Low-grade tumors have a Ki-67 <2%, intermediate-grade tumors have a Ki-67 of 2% to 20%, and high-grade tumors have a Ki-67 >20% (11).

Nucleic acid extraction

DNA and RNA were extracted using standard methods (Qiagen: QIAamp DNA Mini kit and RNeasy Mini kit; Roche: High Pure RNA Paraffin kit). Where nucleic acids were extracted from formalin-fixed paraffin-embedded (FFPE) tissue blocks, 6 to 12 × 6 μm serial sections were cut and macerodissected, ensuring that >80% of the sample was histologically confirmed as tumor. Fresh tissue samples were frozen in liquid nitrogen within 30 minutes of resection. Serial hematoxylin and eosin-stained sections were evaluated to ensure >80% purity of tumor and normal specimens.

DNA mutation analysis: whole-exome and targeted CDKN1B sequencing

Exome sequencing was performed by Joshua Francis on 1 μg DNA as previously described (3). In brief, exonic region capture was performed utilizing the Agilent V2 capture probe set, and sequencing of 76-bp paired end reads was performed utilizing Illumina HiSeq2000 instruments. A median of 9.15 Gb of unique sequence data was generated per sample, which was aligned to the target exome using the Burrows–Wheeler Aligner (BWA), resulting in a median 140× coverage of each base. Targeted sequencing was performed by multiplex PCR reaction tiling of CDKN1B using 100 to 200 ng of DNA on the Fluidigm Access Array microfluidic device. PCR products underwent Illumina sequencing on the MiSeq instrument to a mean coverage of 800×.

DNA methylation analysis: Illumina HumanMethylation450 BeadChip

DNA from SINET samples was analyzed on the HumMeth450 BeadChip (HM450). The integrity and viability of FFPE samples were first determined using the Illumina FFPE QC Kit. Ligation of FFPE DNA (Qiagen REPLI-g FFPE Kit) sample bisulfite conversion of FF and FFPE DNA (Zymo EZ DNA Methylation kit) were performed as described previously (12). Quality control of bisulfite conversion was performed by quantitative PCR to confirm >98% conversion. Data analysis was performed using the ChAMP pipeline (13). Samples where >5% of probes failed detection $P$ value < 0.01 filtering were excluded from further analysis. Normalization with BMIQ and batch correction with ComBat were performed according to pipeline defaults. The comparability of array-based methylation analysis of FFPE and FF tissues has previously been demonstrated (12).

CpG island methylator phenotype (CIMP) was defined by calculating the median methylation score across all CpG island probes on the HM450: CIMP positive: sample demonstrates >5% greater median methylation than median score across all tumors; CIMP negative: >5% less than overall median score. Samples where total CpG methylation score is within 5% of cohort median are unclassified.

DNA methylation analysis: methylation DNA immunoprecipitation sequencing

DNA from 16 tumor and three normal samples underwent whole-genome methylation profiling using methylation DNA immunoprecipitation sequencing (MeDIP-Seq) as previously described (14). Briefly, 1 μg of DNA was sonicated to achieve an average fragment length of 300 bp. Fragmented DNA underwent end repair (NEBNext End Repair Module), dA-tailing (NEBNext dA-Tailing Module), and adapter ligation. DNA purification following each step was performed using Ampure XP magnetic beads (Agencourt). Automated MeDIP was performed according to the Diagenode protocol and utilizing Diagenode-positive and -negative DNA controls. Amplification was performed using NEBNext High Fidelity 2× PCR Master Mix (NEB); size selection was

Translational Relevance

To date, limited understanding of the underlying biology of SINETs has hindered personalized management. The most frequent genetic mutation (CDKN1B, affecting ~8% of tumors) has no immediate detectable impact on clinical phenotype or outcome. Due to their relative rarity, SINETs have not been included in any of the international collaborative cancer molecular profiling efforts. We have therefore performed a Cancer Genome Atlas style—integrated analysis of a large cohort of SINETs in order to comprehensively characterize their molecular profile. We identified three molecular subtypes of SINET that are associated with significant difference in progression-free survival after surgical resection. We also demonstrated that SINETs are highly epigenetically dysregulated and characterized a panel of 21 genes that are epigenetically altered in 70% to 80% of cases. These findings highlight the molecular diversity of SINETs and highlight epigenetic mechanisms as potential drivers of tumorigenesis. Novel molecular profiling could be implemented in the clinical setting to facilitate personalized management and improve prognosis.
performed by gel excision of 300 to 350 base pair fragments. Assessment of fold enrichment of regions of known methylation status was performed. Sequencing was performed on the Illumina HiSeq 2500 in High Output mode (UCL Genomics). MeDIP-seq data were analyzed using the custom pipeline MeDUSA (v2.0; ref. 15). BWA was used to align paired end sequence data to the reference human genome, and filtering was performed to remove reads that were unable to be aligned as a viable pair and also those pairs in which neither read scored an alignment score of >10, resulting in a mean of 59 million unique reads per sample.

Gene expression profiling: Illumina Whole-Genome DASL assay

RNA was assessed using the Agilent Bioanalyzer; samples with RNA concentrations of >40 ng/µl and RNA Integrity Number (RIN) > 1.5 were taken forward for analysis. The whole-genome cDNA-mediated annealing, selection and ligation (WG-DASL; Illumina) assay was performed following the manufacturer's instructions (UCL Genomics). Quantile normalization and background correction were performed in an Illumina GenomeStudio software. Samples where >70% probes failed detection P value < 0.05 filtering were excluded from further analysis. The limma package was used to identify differentially expressed probes (16).

Copy-number variance analysis

Genome-wide copy-number variance was inferred from HM450 signal intensity data within the ChAMP pipeline (17). Whole-chromosome arm copy number was determined using an 80% length threshold and a segment mean threshold of ≥0.2. Analysis of focal copy-number alterations was performed using the GISTIC 2.0 algorithm (18).

Analysis of publicly available data: methylation

Raw methylation data from TCGA cohorts (cancer: colon adenocarcinoma, rectum adenocarcinoma, stomach adenocarcinoma, pancreatic adenocarcinoma; healthy: colon, stomach, rectum, pancreas) were downloaded and analyzed using the Marmal-aid webtool (19). Normalization was performed using the ChAMP pipeline.

Analysis of publicly available data: expression

Affymetrix Human Genome U133 Plus 2.0 Array data were downloaded from the Gene Expression Omnibus (GEO) database, accession code GSE9576 (20). Data processing was performed using GEO2R (21).

Integrated pathway analyses

Pathway analyses were performed on WebGestalt (22).

Availability of data

Raw data from this study will be deposited in GEO (accession number GSE73832).

Results

Genome-wide DNA methylation, gene expression, and copy-number variance (CNV) profiling were performed on 97 SINET tumors (primaries and liver metastases) collected from 85 individuals. A flowchart demonstrating the analyses the samples underwent is shown in Fig. 1. The majority of analyses were performed on 72 primary SINETs with 25 liver metastases utilized as a validation cohort. Of the 72 SINET primary tumor cases, there was a preponderance of females (34 vs. 25 males, gender unknown in 13 cases). Median age at diagnosis was 62 years. Where site of primary within the small intestine was documented, almost all were of ileal origin (40 vs. 4 duodenal/jejunal, 28 not specified). Clinical information regarding Ki-67 and staging was incomplete in some cases, but where available the majority had metastatic disease (28 vs. 9 localized disease), and the majority were low-grade tumors (grade 1: n = 34, grade

![Flowchart demonstrating number of specimens included in each stage of molecular analysis. HM450, HumanMethylation450 BeadChip assay (2 samples failed and were removed from downstream analysis); SCNA, somatic copy-number variance analysis; DASL, whole-genome cDNA-mediated annealing, selection, and ligation assay (1 sample failed and was removed from downstream analysis).](image-url)
2: \( n = 14 \), grade 3: \( n = 2 \); Supplementary Table S2). Twenty-nine cases from the cohort had previously been sequenced for CDKN1B mutations; three of 29 (10%) of these primary SINETs harbored detectable mutations (3).

Novel molecular subgrouping of SINETs

CNV analysis identified recurrent whole-chromosome/whole-arm alterations in SINET primary tumors \( [n = 47] \); chr4 (gain, 19% of cases), chr5 (gain, 11%), chr18 (loss, 64%), and chr20 (gain, 17%), consistent with previous reports (23). Unsupervised hierarchical clustering of whole-chromosome/arm-level CNVs identified three distinct molecular subgroups of SINET primary tumors. Group A harbored chr18 LOH only (18LOH group, 55% of tumors), group B no large copy-number alterations (NoCNV group, 19%), and group C harbored multiple copy-number alterations (including gain of 4, 5, and 20; MultiCNV group, 26%; Fig. 2A). Of note, all three tumors that harbored CDKN1B mutations were located within subgroup A (chr18 LOH) tumors.

Unsupervised analysis determined that the epigenetic profile of SINETs also clearly differentiates the three groups identified by CNV analysis (Fig. 2B). Supervised analysis of 275 genes differentially methylated \( (>30\% \text{ differentially methylated } [\Delta[\beta]], \ P < 0.001) \) between the three subgroups (Fig. 2A; Supplementary Table S1) found that significant differential methylation between subgroups was observed in EGFR, mTOR, and VEGF signaling pathways.

Expression analysis was performed to identify differentially expressed genes between the three subgroups where matched data were available \( (n = 17) \), a panel of 41 probes \( (\text{unadj } P < 0.001) \) was identified which distinguished these groups with 88% accuracy \( (15/17 \text{ cases}; \text{Supplementary Fig. S3}) \). Finally, a CIMP status was defined in SINETs based on overall methylation across all analyzed CpG sites \( (\text{CIMP positive } n = 18, \text{CIMP negative } n = 13) \).

Figure 2.
SINETs may be subdivided into three groups based on copy-number alterations, epigenetic profile, and CDKN1B status. A, three subgroups of SINET primary tumors are identified based on unsupervised hierarchical clustering of whole-chromosome/arm-level somatic copy-number variance profiles. CDKN1B mutations are found only in subgroup A (chr18 LOH). B, unsupervised hierarchical clustering of the methylation status of the top 500 most variable probes across the SINET primary tumor cohort differentiates three subgroups of SINET distinguished by CNV status. C, PFS analysis following surgical resection of primary tumor demonstrates poorer prognosis in the NoCNV and MultiCNV subgroups compared with chr18 LOH subgroup. D, the methylation profile of 21 epigenetically dysregulated genes distinguishes SINET from other GI malignancies and normal GI tissue. Abbreviations: 18LOH, LOH chromosome 18; MultiCNV, multiple arm-level copy-number alterations; NoCNV, no arm-level copy-number alterations; OS, overall survival; TVN, tumor:normal classification.
A significant association between CIMP negativity and chr18 LOH was noted (Fisher exact test, \(P < 0.032\)).

Thirty-two samples classified by molecular subtype were analyzed for PFS following surgical resection of primary tumor. After a median follow-up of 56 months, average PFS across all 32 samples was 40.6 months. Using a Cox proportional hazard model, a difference in the PFS was identified between the subgroups with the 18LOH group having superior PFS (median not reached at 10-year follow-up) when compared with both the NoCNV group (56 months, \(P = 0.10\)) and the MultiCNV group (21 months, \(P = 0.02\); Fig. 1C). Interestingly, the two high-grade tumors in the cohort were both included in the MultiCNV poor prognosis group, however, intermediate- and low-grade tumors did not segregate by subgroup. This indicates that this molecular classification may have utility as an adjunct to Ki-67 in order to refine prognostication. A trend for younger age at diagnosis was identified on comparison of the 18LOH group (median age 67) and the MultiCNV group (median age 54, \(P = 0.06\)). The key characteristics of these molecular subgroups of SINET primary tumors are summarized in Table 1.

### Identification of epigenetic drivers of NET pathogenesis

Methylation profiling of SINET primary tumors (n = 49) identified 130,083 methylation variable positions [MVP; Benjamin–Hochberg (BH) adj \(P < 0.0005\)] differentiating tumor and normal tissues. Of these MVPs, 5,514 (4.2\%) were hypomethylated in tumor by \(>30\%\) and 1,841 (1.4\%) were hypermethylated in tumor by \(>30\%\). KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis of 2,749 differentially methylated genes identified enrichment of multiple cancer-related pathways, including MAPK, Wnt, and PI3K–mTOR signaling pathways (BH adj \(P < 0.0005\); Supplementary Fig. S2).

Gene expression profiling of SINET primary tumors (n = 31) identified 2,016 genes which were differentially expressed between tumor and normal (BH adj \(P < 0.05\)), of these, 1,300 demonstrated a minimum 2-fold average difference in expression. Disease association analysis of these genes identified significant enrichment of intestinal and neoplastic disease processes, as well as endocrine and neurological system disorders.

Integration of DNA methylation and gene expression data identified 61 genes where altered methylation was associated with a change in expression (Supplementary Table S3). Of these genes, 23 were found to be altered in the majority of SINETs. Validation of methylation changes in this panel of 23 candidates was performed by MeDIP-seq of an independent cohort of 16 SI primary NET samples. Validation of altered expression of the 23 candidates was performed using publicly available data (GEO accession number: GSE9576; ref. 20). These confirmatory steps endorsed the validity of 91\% (21 of 23) of the proposed panel of candidate epigenetically regulated genes in SINET pathogenesis (Table 2; Supplementary Fig. S3). Altered methylation in at least 10 of these 21 genes was demonstrated in 65\% of tumor specimens, demonstrating a significant rate of genome alterations. When the top 5 most frequently altered candidates from this panel (caudal type homeobox 1 (CDX1), fructose-1,6-bisphosphatase 1 (FBP1), transmembrane protein 171 (TMEM171), ganglioside-induced differentiation associated protein 1 like 1 (GDAP1L1), and cadherin, EGF LAG seven-pass G-type receptor (CELSR3)) were selected, 82\% of tumor specimens demonstrate altered methylation in at least 4 of the 5 candidates.

Utilizing publicly available TCGA methylation data from other gastrointestinal (GI) tumor samples, we evaluated the methylation status of the panel of 21 candidate genes across a range of tumors. We determined that the methylation signature of this panel robustly distinguished SINETs from other GI malignancies and normal GI tissue (positive predictive value 95.8\%; Fig. 1D).

Of particular interest is hypermethylation of the gastric inhibitory polypeptide receptor gene [GIPR; which was seen in 74\% of SINET (median methylation 0.67 vs. normal 0.29, \(P < 2.2 \times 10^{-16}\))]. To investigate the status of GIPR further, DNA methylation and gene expression profiling were performed on a discrete set of 20 (methylation) and 11 (expression) SINET liver metastases. This confirmed that promoter hypomethylation, gene body hypermethylation (Fig. 3A), and increased expression (Fig. 3B) of GIPR were positively correlated with the development of liver metastases. Finally, through comparison with TCGA data, it was determined that hypermethylation at GIPR was sensitive and specific for the detection of SINET compared with other GI malignancies with an AUC of 0.991 (95\% confidence interval, 0.991–0.999; Fig. 3C). Of note, in addition to GIPR, two further G protein–coupled receptor genes (prolactin releasing hormone receptor (PRLHR) and CELSR3) and three enzymes [pancreatic lipase related protein 2 (PNLIPRP2), protein tyrosine phosphatase receptor type N (PTPRN), and proprotein convertase subtilisin/kexin type 1 (PCSK1)] represent potentially druggable targets within the 21-gene panel.

### Table 1. Characteristics of three molecular subgroups of SINET primary tumors

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumors, n (% of total)</td>
<td>26 (55%)</td>
<td>9 (19%)</td>
<td>12 (26%)</td>
</tr>
<tr>
<td>CNV profile</td>
<td>18LOH</td>
<td>No CNV</td>
<td>Multiple CNV (18LOH and amplifications)</td>
</tr>
<tr>
<td>Median arm-level CNVs per tumor (range)</td>
<td>2 (1–6)</td>
<td>0 (0–0)</td>
<td>6 (2–11)</td>
</tr>
<tr>
<td>CDKNIB mutation rate (% of total)</td>
<td>3 mut (11.5%)</td>
<td>0 mut (0%)</td>
<td>0 mut (0%)</td>
</tr>
<tr>
<td>CIMP positive</td>
<td>8 (30.8%)</td>
<td>5 (55.6%)</td>
<td>5 (41.7%)</td>
</tr>
<tr>
<td>CIMP negative</td>
<td>11 (42.3%)</td>
<td>1 (3.3%)</td>
<td>1 (8.3%)</td>
</tr>
<tr>
<td>Median age at diagnosis, y</td>
<td>67</td>
<td>60</td>
<td>54</td>
</tr>
<tr>
<td>Stage, metastatic (%)</td>
<td>8 (30.8%)</td>
<td>3 (33.3%)</td>
<td>5 (41.7%)</td>
</tr>
<tr>
<td>Stage, localized (%)</td>
<td>3 (11.5%)</td>
<td>2 (22.2%)</td>
<td>5 (36.7%)</td>
</tr>
<tr>
<td>PFS after resection (months)</td>
<td>Not reached</td>
<td>56</td>
<td>21</td>
</tr>
</tbody>
</table>

NOTE: Three novel molecular subgroups of SINET are defined by distinct copy number and epigenetic profiles, with prognostic impact on PFS following resection of primary tumor.
Epigenetically dysregulated genes on chromosome 18

The most common subgroup of SINETs was group A chr18 LOH (26/47, 55% of our cohort). Historically, this has also been the most frequently described molecular alteration in these tumors; however, tumorigenic drivers from this region have not been identified to date. We therefore interrogated chr18 to identify epigenetic changes which may represent the “second hit” following LOH. In total, 1,043 probes on chr18 demonstrated differential methylation (P < 0.05; Δβ >10%) between tumors affected by chr18LOH and normal SI (Supplementary Table S4). Furthermore, 25 genes were found to be differentially expressed (adjP < 0.05) between chr18LOH tumors and normal SI. From this list, several genes on chr18 where epigenetic changes were associated with reduced expression were identified including: laminin alpha 3 (LAMA3), serpin peptidase inhibitor clade B member 5 (SERPINB5), and tumor necrosis factor receptor superfamily member 11a NFkB activator (RANK or TNFRSF11A).

Discussion

Here, we present the first comprehensive integrated molecular analysis of SINETs, characterizing DNA mutation, methylation, and RNA expression profiles of a large cohort of tumors. Despite being a relatively small and heterogeneous cohort when compared with other TCGA-type studies, it comprises the largest integrated genomic analysis in this tumor type.

We have identified three subgroups of SINET distinguished by molecular profiling. The largest group is defined by chr18LOH and is associated with the presence of CDKN1B mutations, and CIMP negativity. Patients classified within this subgroup have the most favorable PFS (not reached at 10-year follow-up) after resection and an older age at diagnosis suggesting a less aggressive phenotype. A second subgroup is characterized by the absence of arm-level CNVs and is associated with a high level of CIMP positivity and an intermediate PFS (56 months). The final subgroup comprises 26% of tumors and is characterized by the presence of multiple CNVs, a significantly poorer PFS (21 months) and younger age at onset suggesting a more aggressive clinical phenotype. The prognostic impact of these newly defined subgroups requires validation in an independent cohort; however, we suggest that these novel molecular classifications may be utilized as an adjunct to Ki-67 to optimize accurate prognostication for patients. Furthermore, SINETs demonstrating the MultiCNV profile may be considered for trials of more aggressive management.

The association between chr18LOH and CIMP negativity suggests that aberrant methylation plays an even greater role in tumors lacking the characteristic chr18 LOH, and is supported by previously published data in SINET and colorectal adenocarcinoma (24, 25).

We have demonstrated that SINETs are epigenetically dysregulated tumors and are therefore may be candidates for trials of novel targeted epigenetic therapies. This is in keeping with prior molecular analyses of pancreatic and bronchial NETs demonstrating epigenetic dysregulation in these populations. A panel of 21 epigenetically dysregulated genes has been identified and validated. Epigenetic changes affecting this panel appear to be specific to SINETs with respect to gastrointestinal adenocarcinomas. In particular, aberrant methylation of GIPR is proposed as a mechanism of upregulation of this gene. GIPR overexpression in...
SINETs has been reported previously, and radiolabeled ligands are in development as novel diagnostic agents for NETs particularly those not expressing somatostatin receptors (26); however, this is the first description of aberrant methylation being associated with GIPR upregulation in NETs. The success of novel radioligands suggests that therapeutic agents targeted to the GIPR receptor could be developed and may increase the range of molecularly targeted therapeutics available for use in NET patients. The CDX1-encoded transcription factor plays a key role in intestinal differentiation, and CDX1 has been identified in both colorectal and gastric cancers as a tumor suppressor gene where downregulation is secondary to hypermethylation (27). FBP1 undergoes promoter hypermethylation in colorectal cancer and hepatocellular carcinoma but has not previously been associated with NETs (28). These are examples of genes within the 21-gene panel, which represent epigenetically dysregulated downregulated genes in SINETs.

Finally, we have identified genes found on chromosome 18 which are affected by a "second hit" of epigenetic dysregulation following LOH of this region. LAMA3 hypermethylation has not previously been described in NETs but is a feature of gastric adenocarcinoma, prostate cancer, and breast cancer, and in the case of breast and prostate cancers, hypermethylation of the gene is associated with poorer clinical outcomes (29–31). SERPINB5 has been described as a "metastasis suppressor gene," and hypermethylation of this gene is associated with metastatic potential in pancreatic adenocarcinoma and poorer clinical outcomes in breast cancer (32, 33). Methylation of SERPINB5 in NETs has previously been demonstrated by Verdugo and colleagues; however, determination of hypermethylation compared with normal tissue was not performed (34).

This study has been performed as a comprehensive basic molecular profiling experiment. It is therefore limited by a lack of functional validation studies. In SINETs, such studies are difficult to perform due to the lack of validated cell lines or animal models of these tumors. These data remain exploratory at this stage and require further validation in a separate cohort. This would allow for external validation of the panel of 21 genes identified in this study as the BH FDR approach used allows for some false-positive results.
External validation of methylation profiling would be preferred; however, there are no publicly available comparable datasets, emphasizing the importance of all investigators making available results of molecular studies in rare tumors such as SINETs.

In summary, we have performed the largest unbiased integrated genomic, epigenomic, and transcriptomic profile of small intestinal NETs yet reported. The findings presented here form the basis for a novel molecular classification of SINET tumors in the clinical setting and underscore the feasibility of stratifying NET patients by molecular features. These mutationally quiet tumors have abundant epigenetic changes, which include putative drivers of tumorigenesis.

**Disclosure of Potential Conflicts of Interest**

M. Meyerson reports receiving a commercial research grant from Bayer, and has ownership interest (including patents) in and is a consultant/advisory board member for Foundation Medicine. C. Thirlwell is a consultant/advisory board member for Ipsen. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Karpathakis, H. Dibra, J. Francis, D. Mandair, C. Thirlwell

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Karpathakis, T. Morris, M. Novelli, S.L. Asa, C. Thirlwell

Writing, review, and/or revision of the manuscript: A. Karpathakis, C. Pipinikas, A. Feber, T. Morris, S. Serra, O. Oggunbiyi, T.V. Luong, S.L. Asa, M. Kulke, C. Toumpanakis, T. Meyer, M. Caplin, C. Thirlwell

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Dibra, C. Pipinikas, J. Francis, D. Oukrif, M. Mohmadouloev, S. Serra

Study supervision: C. Thirlwell

Other (histopathology/immunohistochemistry analysis and interpretation): M. Novelli

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