Global DNA methylation patterns through an array-based approach in small intestinal neuroendocrine tumors

Dear Editor

Small intestinal neuroendocrine tumors (SI-NET; formerly midgut carcinoid) are rare hormone-secreting tumors, which develop from the enterochromaffin cells in the gastrointestinal tract and have an annual incidence of ~2 per 100,000 individuals (Modlin et al. 2003). The genetic background of SI-NETs is poorly characterized, although a few recurrent numerical imbalances have been found (Kytola et al. 2001, Lollgen et al. 2001). Loss of chromosome 18 and deletions of 18q21-qter and 11q22–q23 are most prevalent in SI-NETs, however, no recurrent mutation has been found in these regions.

Epigenetic alterations play an important role in tumorigenesis, of which DNA methylation in CpG islands is best characterized. Promoter hypermethylation surrounding transcription start sites correlates with transcriptional silencing (Eckhardt et al. 2006). In contrast, global hypomethylation is a common phenomena in cancer and increases mutation rates (Chen et al. 1998) and chromosomal instability (Eden et al. 2003). Promoter methylation of RASSF1A and CTNNB1 may play a role in tumor progression and metastatic transformation in SI-NETs (Zhang et al. 2006). Hypermethylation of p14 in 4/7 SI-NETs compared with normal adjacent mucosa, and global hypomethylation of long interspersed elements (LINE1) and ALU repetitive elements correlating to loss of chromosome 18 and lymph node metastases have been reported (Chan et al. 2003, Choi et al. 2007).

We have investigated the DNA methylation status of matched primary SI-NETs and their mesenteric lymph node metastases (n=20) using a commercially available array. All patients included in this study were diagnosed with metastatic SI-NETs and tumor specimens were collected immediately after routine surgical procedure at Uppsala University Hospital and were histopathologically confirmed. Informed written consent and approval of local ethical committee was obtained before this study. Bisulfite-treated tumor DNAs were hybridized to the HumanMethylation27 Beadchip (Illumina, San Diego, CA, USA) with the whole-genome covering 27,578 CpG loci located in 14,495 genes. The methylation level was determined by the fluorescence signal C (methylated) assigned 1 and T (unmethylated) assigned 0. The β-value was calculated by the relative value of the fluorescence signal between C and the total fluorescence signal at a single locus given a value ranging from 0 to 1, where 1 reflects a methylated locus. Genome studio methylation module was used for data analysis. Genome studio analyzes the β-value for each CpG locus, and intensity for each probe, and calculates the P value for each measurement. The samples were divided into two groups: primary tumors (n=10) and lymph node metastases (n=10). To find differentially methylated genes between the primary tumors and metastatic tumors, differential methylation analysis was performed. We defined methylation as a β-value of 0.7 or above (Kanduri et al. 2010). Highly methylated gene value was defined as a β-value of 0.9 or above. Investigated CpG loci in the metastases group were significantly less methylated, with an average β-value of 0.2576, compared with primary tumors having an average β-value of 0.2608, which indicates global low methylation in SI-NETs. These results are in accordance with a previous report (Choi et al. 2007). Hypomethylation might contribute to malignant behavior through genomic instability (Masramon et al. 2006).

We have identified in total 2697 CpG sites significantly differentially methylated (P>0.05) when comparing the primary tumors with corresponding metastases. Furthermore, we classified the top 120 genes with significant differential methylation and grouped them into functional classes. A large number of genes regulating cell growth, apoptosis, proliferation, and metastases-related genes were found to be differentially...
methylated. This reflects the complex epigenetic machinery that takes part during metastatic progression.

The tumor suppressors RUNX3, TP73, and CHF8, known to be epigenetically silenced in other cancer types (Geli et al. 2008), were highly methylated (β-value ≥ 0.9) in the majority of the SI-NETs. Other genes previously reported to be hypermethylated in different cancer types were also amongst the most methylated genes in our cohort; MAPK4, CCND1, AHR8R, and RB (RB1).

Previous studies on loss of heterozygosity have identified loss of chromosome 18, 18q21-qter, and 11q22–q23, but no recurrent mutations have yet been found. All methylated genes (β-value > 0.7) at 18.21-qter and 11q22–q23 were identified. At chromosome 18q21-qter, SETBP1, ELAC1, MB1, MAPK4, and TCEB3C were highly methylated in all SI-NETs included in our cohort. Several members in the Serpin peptidase inhibitor family were also methylated (e.g., SERPINB3, SERPINB5). ARVC1, MMP8, BTG4, APOA1, FAM89B, and HSPB1 at 11q22–q23 were methylated in all tumors, while HTR3B, CD3D, and TRIM29 were highly methylated in the vast majority of the SI-NETs.

Several candidate tumor genes can be identified for future investigations from the results described above. Methyl-CpG binding domain protein 1 (MBD1) represses transcription of genes with methylated promoters and depletion of MBD1 in vivo resulted in a more invasive and migratory phenotype (Yaqinuddin et al. 2008). The apoptosis-related gene BCL2 is hypermethylated in ~50% of prostate cancer (Cho et al. 2007) and in our cohort was highly methylated in 80% of the tumors. BTG4 at 11q23 is hypermethylated in gastric cancer and its expression is restored by 5′-aza-2′-deoxycytidine treatment (Dong et al. 2009).

Another five genes (AXL, CRM1P1, FGF5, CXXC5, and APOBEC3C) with statistically significant differential methylation levels in primary tumors and metastases were selected and their mRNA expression levels were analyzed in an extended cohort (n = 47); primary tumors (n = 9) were matched with the corresponding lymph node metastases (n = 9) and liver metastases (n = 9), another set of matched primary tumors (n = 9) with lymph node metastasis (n = 9) and in addition two primary tumors with no matched metastases, using commercially available probe-based gene expression analysis assays (Applied Biosystems). We used 2⁻ΔΔCt to calculate the relative gene expression levels, with 18S rRNA as internal standard and JMP 8.0 (SAS Institute, Cary, NC, USA) for statistical analysis. Unpaired t-test was used for all analyses and a P<0.05 was considered statistically significant. The relative mRNA expression level of the differentially methylated genes AXL, CRM1P1, FGF5, and APOBEC3C, but not of CXXC5 largely reflected the methylation status.

Unsupervised hierarchical clustering of the samples identified three distinct clusters denoted A, B, and C (Fig. 1). Cluster C showed a higher methylation index and included the aggressive phenotype. The tumors had a higher Ki67 index, patients were older at the time of diagnosis and included a higher level of chromogranin A (CgA) 1-year postoperatively than the other patients (Table 1). The cluster analysis thus separated the two patients with most aggressive phenotype in the cohort, both patients having disseminated disease. Further conclusions are limited due to the small number of patients included in the study.

In summary, our data supports the possibility of a role for aberrant DNA methylation during metastatic

### Table 1 Clinical characteristics of the three SI-NETs clusters

<table>
<thead>
<tr>
<th>Cluster group</th>
<th>Number of patients</th>
<th>β-value</th>
<th>Mean age at diagnosis (s.e.m.)</th>
<th>Ki67 (%)</th>
<th>Mean survival (months)</th>
<th>Mean follow-up (months)</th>
<th>CgA 1-year postoperative</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>n = 5</td>
<td>0.25 ± 0.001</td>
<td>52 ± 5.7</td>
<td>1% ± 0</td>
<td>Alive</td>
<td>51 ± 19.3</td>
<td>15 ± 9.4</td>
</tr>
<tr>
<td>B</td>
<td>n = 3</td>
<td>0.269 ± 0.001</td>
<td>58 ± 10</td>
<td>1% ± 0</td>
<td>Alive</td>
<td>81 ± 8.7</td>
<td>19 ± 14.4</td>
</tr>
<tr>
<td>C</td>
<td>n = 2</td>
<td>0.27 ± 0.001</td>
<td>75 ± 1</td>
<td>13.5% ± 10.8</td>
<td>40.2 ± 4.3</td>
<td>72 ± 23</td>
<td></td>
</tr>
</tbody>
</table>

All data are presented as mean ± s.e.m. CgA, chromogranin A.
progression of SI-NETs. We have identified several highly methylated genes of special interest for further analysis. Unsupervised clustering identified three distinct SI-NET subgroups. Interestingly, a subgroup of patients with a more malignant behavior was classified into a separate cluster. To our knowledge this is the first time aggressive behavior of SI-NETs could be predicted by their DNA methylation signature; however, future studies have to confirm this finding in a larger cohort.

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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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