

# Global hypomethylation and promoter methylation in small intestinal neuroendocrine tumors

## An in vivo and in vitro study

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**Keywords:** neuroendocrine, carcinoid, SI-NET, DNA methylation, pyrosequencing

Aberrant DNA methylation is a feature of human cancer affecting gene expression and tumor phenotype. Here, we quantified promoter methylation of candidate genes and global methylation in 44 small intestinal-neuroendocrine tumors (SI-NETs) from 33 patients by pyrosequencing. Findings were compared with gene expression, patient outcome and known tumor copy number alterations. Promoter methylation was observed for *WIF1*, *RASSF1A*, *CTNNB1*, *CXCL14*, *NKX2-3*, *P16*, *LAMA1*, and *CDH1*. By contrast *APC*, *CDH3*, *HIC1*, *P14*, *SMAD2*, and *SMAD4* only 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*, *NKX2-3*, and *CXCL14* expression was reduced in metastases vs. primary tumors ( $P < 0.02$ ). Low expression of *RASSF1A* and *P16* were associated with poor overall survival ( $P = 0.045$  and  $P = 0.011$ , respectively). Global methylation determined by pyrosequencing of LINE1 repeats was reduced in tumors vs. normal references, and was associated with loss in chromosome 18. The tumors fell into three clusters with enrichment of *WIF1* methylation and LINE1 hypomethylation in Cluster I and *RASSF1A* and *CTNNB1* methylation and loss in 16q in Cluster II. In Cluster III, these alterations were low-abundant and *NKX2-3* methylation was low. Similar analyses in the SI-NET cell lines HC45 and CNDT2 showed methylation for *CDH1* and *WIF1* and/or *P16*, *CXCL14*, *NKX2-3*, *LAMA1*, and *CTNNB1*. Treatment with the demethylating agent 5-azacytidine reduced DNA methylation and increased expression of these genes in vitro. In conclusion, promoter methylation of tumor suppressor genes is associated with suppressed gene expression and DNA copy number alterations in SI-NETs, and may be restored in vitro.

### Introduction

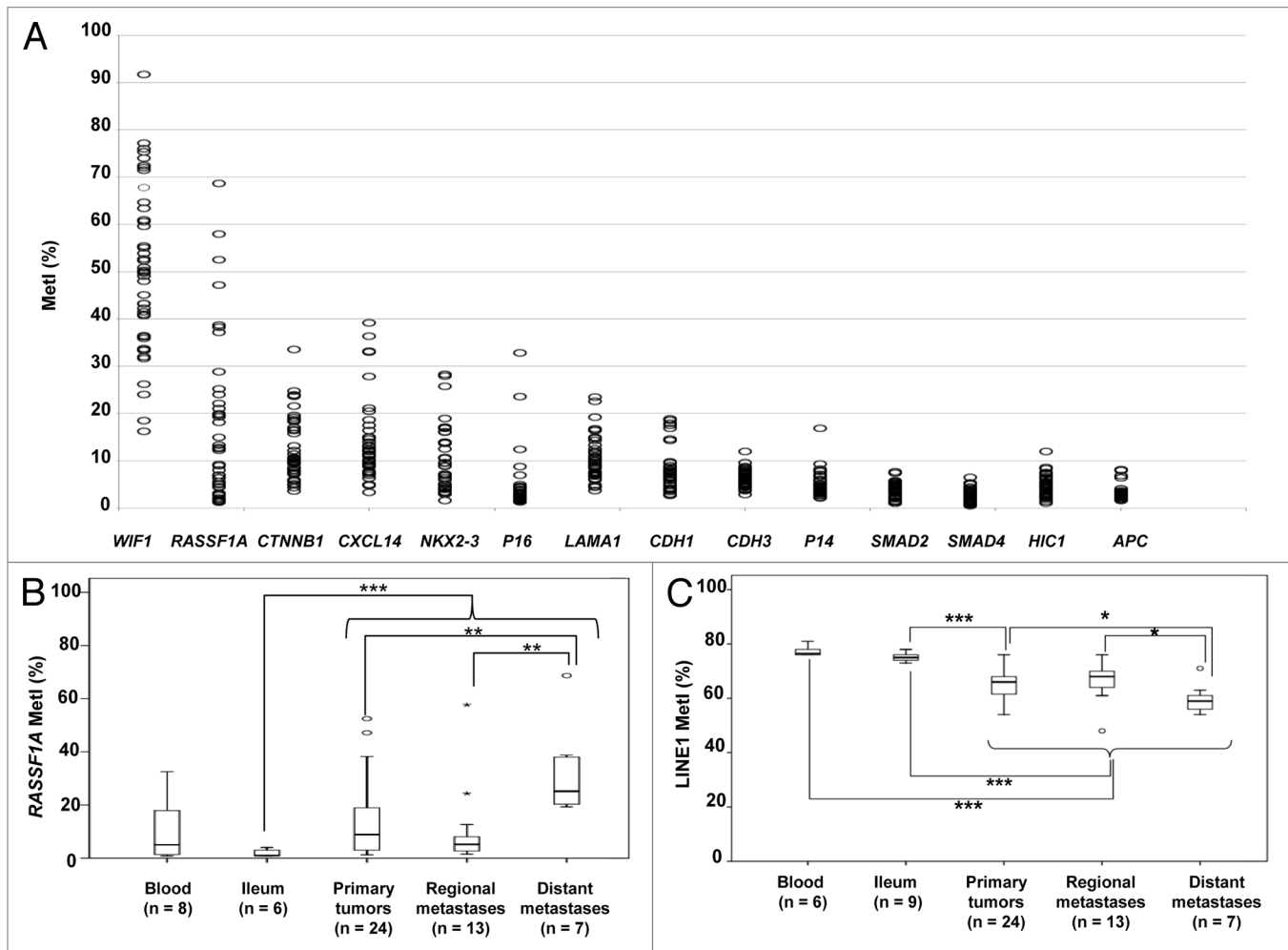
Small intestinal neuroendocrine tumors (SI-NET) originate from enterochromaffin cells in the ileal mucosa. The incidence of the disease has been increasing and is presently more than 1.1 per 100000.<sup>1,2</sup> Based on genome-wide studies, a few recurrent regions of copy number alterations have been identified.<sup>3</sup> Most commonly, this involves loss in chromosome 18;<sup>4</sup> however, no target gene has so far been precisely identified. Genes located within recurrently altered chromosomal regions, along with deregulated genes reported in expression profiling studies,<sup>5</sup> are candidates for being involved in the tumor development.

Studies of epigenetic modifications, predominantly DNA methylation, may further reveal aberrations contributing to tumor development. Aberrant methylation of promoter CpG islands with effects on gene expression has been linked to many

cancer types. Moreover, global genomic hypomethylation may contribute to genomic instability, a characteristic of human cancer.<sup>6</sup> Global methylation may be assessed by different approaches, for example, by analysis of long interspersed element 1 (LINE1), which constitutes 17% of the human genome<sup>7</sup> and has also been linked to genomic stability.<sup>8</sup> In SI-NETs, several genes have been shown to be methylated using qualitative methodologies, e.g., *RASSF1A* and *CTNNB1*.<sup>9,10</sup> Global hypomethylation of LINE1 and Alu repeats has been demonstrated by quantitative pyrosequencing in a limited number of SI-NETs.<sup>11</sup> Recently, a genome-wide profiling study reported several methylated genes in SI-NETs with a general tendency to relative hypomethylation in lymph node metastases compared with primary tumors.<sup>12</sup> However, little is known about tumor-specific alterations, the relationship between methylation density and gene expression in tumors, and whether the alterations may be reversed in vitro.

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Submitted: 01/03/2014; Revised: 04/11/2014; Accepted: 04/16/2014; Published Online: 04/24/2014  
<http://dx.doi.org/10.4161/epi.28936>



**Figure 1.** Results from quantifications of gene specific promoter methylation global methylation. **(A)** Scatter plot showing MetI values (mean methylation of the CpGs assessed in %) for each gene promoter in individual samples of SI-NETs. **(B)** Comparison of *RASSF1A* methylation in normal references, primary tumors and regional and distant metastases. **(C)** LINE1 global methylation in the different sample groups. Outliers are indicated by open circles and extremes by asterisks. *P* values for statistical comparisons between groups are indicated for suggestive *P* values  $\leq 0.050$  (\*) or  $\leq 0.01$  (\*\*), and for significant *P* values  $\leq 0.001$  (\*\*\*)

In this study, promoter methylation densities were quantified in a panel of SI-NETs and normal references for candidate tumor suppressor genes located in chromosomal regions with recurrent copy number losses or showing downregulation in expression profiling studies. Global methylation was assessed for LINE1 repeats and by ELISA-based detection of 5-methyl cytosines. The expression of methylated genes was further investigated in vitro after treatment with a demethylating agent. Associations to global DNA methylation, DNA copy number status, tumor characteristics and patient outcomes were also investigated.

## Results

### Gene-specific promoter methylation in SI-NETs

We quantified promoter methylation of 14 candidate genes in a panel of SI-NETs, together with blood and normal ileum

used as references (Table 1). The genes were selected based on known tumor suppressor function and reported aberrant methylation in neuroendocrine tumors (*RASSF1A*, *CTNNB1*, *P16*, *P14*, *HIC1*, and *APC*),<sup>9,10,13</sup> other tumors (*WIF1*),<sup>14</sup> location within frequently deleted regions in SI-NETs (*CDH1*, *LAMA1*, *CDH3*, *SMAD2*, *SMAD4*),<sup>15</sup> or downregulation in SI-NETs (*CXCL14* and *NKX2-3*).<sup>5</sup> Eight genes were found methylated in SI-NETs with frequent individual methylation indices (MetI)  $> 10\%$ , including *WIF1*, *RASSF1A*, *CTNNB1*, *CXCL14*, *NKX2-3*, *P16*, *LAMA1*, and *CDH1* (Fig. 1A; Table S1). The highest methylation in SI-NETs was detected for *WIF1*, with a mean MetI of 50% (range 16–92%). By contrast, the mean MetI was  $<10\%$  (with individual MetI  $> 10\%$  rarely observed) for *APC*, *CDH3*, *HIC1*, *P14*, *SMAD2*, and *SMAD4*, and they were therefore excluded from further analysis (Fig. 1A).

Comparison of MetIs between tumor groups revealed increased methylation of *RASSF1A* in distant metastasis vs. primary tumors ( $U = 325$ ,  $P = 0.005$ ) and vs. regional metastasis

**Table 1.** Summary of results from gene specific promoter and global methylation quantification

Gene symbol	Chromosomal location	Tumors MetI		Normal Ileum MetI		Blood MetI	
		n =	mean (range) %	n =	mean (range) %	n =	mean (range) %
<b>Gene specific promoter methylation</b>							
<i>WIFI</i>	12q14.3	44	50 (16–92)	9	49 (27–75)	8	25 (20–30)
<i>RASSF1A</i>	3p21.31	44	16 (1–69)	6	2 (1–4)	8	11 (1–33)
<i>CTNNB1</i>	3p22.1	44	13 (4–34)	9	14 (4–30)	8	8 (5–11)
<i>CXCL14</i>	5q31.1	44	14 (3–39)	9	14 (10–20)	7	14 (11–16)
<i>NKX2–3</i>	10q24.2	44	10 (2–28)	9	10 (6–16)	7	5 (1–8)
<i>P16 (CDKN2A)</i>	9p21.3	41	4 (1–33)	5	2 (1–4)	4	2 (1–3)
<i>LAMA1</i>	18p11.31	44	10 (4–24)	9	15 (8–22)	8	14 (9–21)
<i>CDH1</i>	16q22.1	44	8 (3–22)	9	11 (5–20)	8	22 (13–29)
<i>CDH3</i>	16q22.1	44	6 (3–12)	9	9 (4–29)	8	9 (7–12)
<i>P14 (CDKN2A)</i>	9p21.3	42	5 (2–17)	5	5 (4–6)	4	3 (2–3)
<i>SMAD2</i>	18q21.1	44	4 (1–8)	9	5 (2–15)	7	6 (4–8)
<i>SMAD4</i>	18q21.2	44	3 (1–6)	9	3 (1–8)	7	4 (2–5)
<i>HIC1</i>	17p13.3	44	5 (1–12)	9	9 (2–19)	7	12 (5–19)
<i>APC</i>	5q22.2	41	3 (2–8)	5	5 (4–8)	4	4 (3–6)
<b>Global methylation</b>							
LINE1		44	65 (48–76)	9	75 (73–78)	6	77 (76–81)
ELISA		27	1.1 (0.32–1.83)	8	1.2 (0.69–1.93)	-	-

Met I, Methylation Index.

( $U = 9$ ,  $P = 0.004$ ) (Fig. 1B). For *WIFI*, *CTNNB1*, *CXCL14*, *NKX2-3*, *P16*, *LAMA1*, and *CDH1* no differences in MetI values were observed between tumor groups ( $P > 0.05$ ). Some of these eight genes were also found to be methylated in normal ileum and/or blood samples (Table 1). As compared with reference samples, statistically significant higher methylation was found for *WIFI* in SI-NETs vs. blood ( $U = 22$ ,  $P < 0.001$ ), and for *RASSF1A* in tumors vs. normal ileum ( $U = 21$ ,  $P = 0.001$ ). For comparison, in the two tumor samples from the colorectal carcinoma case, none of the 14 analyzed genes showed increased methylation as compared with blood and ileum (data not shown).

For the eight methylated gene promoters, we associated MetI with patient gender, age at diagnosis, size of the primary tumor, and presence and type of metastasis. This revealed higher *RASSF1A* methylation in SI-NETs from female (mean MetI 21%; range 3–69%) vs. male cases (mean MetI 8%; range 1–29%) ( $U = 113$ ,  $P = 0.004$ ). To further investigate this difference, pooled normal blood DNA from 10 unaffected males and 10 unaffected females were compared and showed that *RASSF1A* MetIs were similar in female and male blood samples (18% and 15%, respectively). No correlations were detected between MetIs and clinical characteristics for the other seven genes.

#### Global hypomethylation in SI-NETs

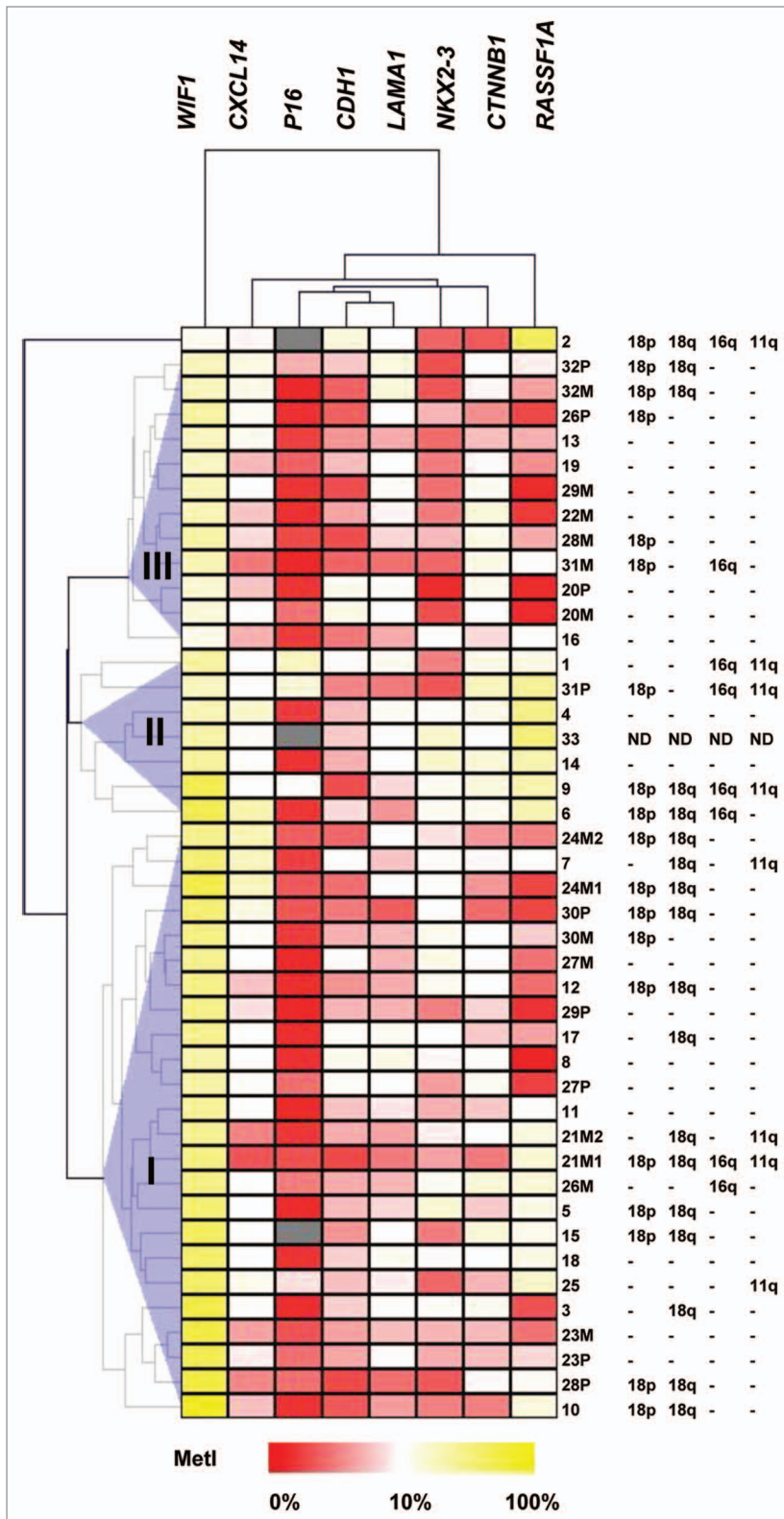
Overall, SI-NETs showed significantly lower LINE1 MetI (54–76%) than normal ileum ( $U = 20$ ,  $P < 0.001$ ) and reference blood ( $U = 0$ ,  $P < 0.001$ ). Lower LINE1 methylation was observed in distant metastasis compared with primary tumors

( $U = 41$ ,  $P = 0.041$ ) or regional metastasis ( $U = 18$ ,  $P = 0.029$ ) (Fig. 1C). The finding of global hypomethylation in SI-NETs was also verified using an ELISA-based methodology with 5-methyl cytosine antibodies (Table 1). Statistically significant associations to patient characteristics were not revealed.

#### Correlations and clustering of gene-specific and global methylation

We compared the results from promoter methylation analyses with global methylation measurements using Spearman rank order analysis. While LINE1 methylation was significantly correlated with *CDH1* and *LAMA1* methylation ( $r = 0.574$ ,  $P < 0.001$  and  $r = 0.510$ ,  $P < 0.001$ , respectively), LINE1 methylation was significantly inversely correlated with methylation of *WIFI* ( $r = -0.470$ ,  $P < 0.001$ ) and inversely correlated with *RASSF1A* methylation ( $r = -0.292$ ,  $P = 0.026$ ) (Table S2). Global methylation examined by ELISA also showed a positive correlation with *LAMA1* methylation ( $r = 0.498$ ,  $P = 0.002$ ) and an inverse correlation with methylation of *WIFI* ( $r = -0.491$ ,  $P = 0.003$ ).

Three clusters were identified from unsupervised hierarchical clustering of the eight methylated genes (Fig. 2). The clusters were characterized by the following differences ( $P < 0.05$ ): *WIFI* had higher MetIs in Cluster I than Cluster II and III; *RASSF1A* and *CTNNB1* showed higher MetIs in Cluster II than in I or III; *NKX2-3* methylation was lower in Cluster III compared with I and II and; Cluster I had lower global methylation by ELISA than Clusters II and III, and lower LINE1 methylation compared with Cluster III.



**Figure 2.** Unsupervised hierarchical clustering of Pyrosequencing data for genes that were found methylated in SI-NETs. MetI values of 0–100% were converted to a range of 0 to 1 and subjected to Euclidean hierarchical clustering. The three tumor clusters are indicated as I, II and III. Gray indicates lack of data. Previously published<sup>15</sup> data for copy number loss determined by qPCR for loci representing 11q (*SDHD*), 16q (*CDH1*), 18p (*EMILIN2*) and 18q (*CDH19*) in case 1–32 are indicated to the right for comparison.

### Association of MetI with DNA copy number aberrations

Results from methylation analysis were compared with DNA copy numbers based on TaqMan copy number analysis of loci representing chromosomal regions 11q, 16q, 18p, and 18q<sup>15</sup> published for case 1–32 in the same tumor series. We found that low levels of *LAMA1* methylation were associated with loss in 18p ( $U = 146$ ,  $P = 0.045$ ). Furthermore, low global LINE1 methylation was associated with loss in 18p and 18q ( $U = 134$ ,  $P = 0.022$  and  $U = 92$ ,  $P = 0.003$ , respectively).

With respect to the three identified clusters, loss in 18q was more frequent in Cluster I than in Cluster III ( $P = 0.030$ ), 11q loss occurred more often in Cluster II compared with Cluster III ( $P = 0.025$ ), and Cluster II included more samples with loss on 16q compared with Clusters I and III ( $P = 0.007$  and  $P = 0.022$ , respectively).

### mRNA expression levels of methylated genes in SI-NETs

Relative mRNA expression levels were determined for the methylated genes *WIF1*, *RASSF1A*, *CTNNB1*, *CXCL14*, *NKX2-3*, *PI6*, *LAMA1*, and *CDH1* using TaqMan-based qRT-PCR and evaluated against MetI values and between sample groups. Overall, statistically significant correlations were not observed between mRNA levels and MetI values of individual genes in SI-NETs. As compared with the normal references, *WIF1* expression was lower in tumors compared with normal ileum ( $U = 60$ ,  $P = 0.003$ ). In addition, several genes showed differential expression levels between tumor sample groups. Lower expression was observed for *WIF1* in metastases compared with primary tumors ( $U = 118$ ,  $P = 0.004$ ); for *CXCL14*, in metastasis compared with primary tumors ( $U = 138$ ,  $P = 0.016$ ); for *CXCL14*, in distant metastasis compared with all other tumor groups ( $U = 51$ ,  $P = 0.013$ ); and for *NKX2-3*, in distant metastasis compared with the other tumor groups ( $U = 45$ ,  $P = 0.008$ ) (Fig. 3). In contrast, increased expression was revealed for *CTNNB1* in metastases compared with primary tumors ( $U = 146$ ,  $P = 0.041$ ); for *PI6*, in distant metastasis in comparison to other tumors ( $U = 52$ ,  $P = 0.015$ ); and for *RASSF1A*, in regional metastasis compared with the other tumors ( $U = 94$ ,  $P = 0.008$ ) (Fig. 3).

*RASSF1A* expression was associated with global methylation by ELISA ( $r = 0.423$ ,  $P = 0.011$ ), while *PI6* expression was negatively associated with LINE1 methylation ( $r = -0.382$ ,  $P = 0.006$ ).

Comparison to clinical findings revealed that *CTNNB1* expression was correlated with tumor size ( $r = 0.339$ ,  $P = 0.035$ ) and tumor stage and did not have a confounding effect on this correlation.

Associations between gene expression levels and gender, age at diagnosis, or metastasis were not observed. The association that was found between gender and *RASSF1A* methylation was not observed for the corresponding mRNA expression.

#### Survival analysis

Gene-specific promoter methylation or global methylation levels were not found to influence patient outcome in terms of survival. However, low mRNA expression levels of *RASSF1A* and *P16* were each associated with short survival ( $P = 0.045$  and  $P = 0.011$ , respectively) (Fig. 4). In multivariate analysis, the clinical variables gender, tumor size and age at diagnosis did not affect the survival function of *RASSF1A* expression. However, age at diagnosis had an impact on the association between *P16* expression and survival ( $\beta = 0.071$ ,  $P = 0.034$ ).

#### Restoration of mRNA expression after demethylation in vitro

To investigate the effect of DNA methylation on gene expression in vitro, the SI-NET cell lines HC45 and CNDT2 were treated with the demethylating agent 5-azacytidine (5-aza-CR) for 4 days. Before treatment MetIs > 20% were revealed for *WIFI*, *P16*, *CXCL14*, *NKX2-3*, *CDHI*, *LAMAI*, and *CTNNB1* in CNDT2 cells and for *CDHI* and *WIFI* in HC45 cells (Table S1); these methylation levels were reduced ( $P \leq 0.050$ ) after 5-aza-CR treatment in both cell lines (Fig. 5). In CNDT2 cells, 5-aza-CR treatment was associated with increased gene expression of *WIFI*, *P16*, *CDHI*, *LAMAI*, and *CTNNB1*. *RASSF1A* expression was also increased; however, the methylation level was constitutionally low and could therefore not be affected by 5-aza-CR. In HC45 cells, 5-aza-CR treatment led to lower promoter methylation density for *CDHI* and *WIFI*, as well as corresponding increase in gene expression levels ( $P \leq 0.050$ ). We also observed increased expression of *CTNNB1*, *P16*, and *RASSF1A* and reduced expression of *CXCL14* and *LAMAI* after 5-aza-CR treatment; however, the corresponding methylation levels were low both before and after treatment (Fig. 5). Global LINE1 methylation was reduced after treatment with 5-aza-CR in both cell lines (data not shown).

## Discussion

Here we demonstrate global hypomethylation and gene-specific promoter methylation in SI-NETs using a quantitative approach by pyrosequencing.<sup>16</sup> For some genes, differential methylation as well as corresponding mRNA expression was observed between tumor groups and in association with survival. Methylation could be reverted and expression restored upon treatment with a demethylating agent in vitro. Applying a cut-off of 10% for biologically significant promoter methylation we found promoter methylation for *WIFI*, *RASSF1A*, *CTNNB1*, *CXCL14*, *NKX2-3*, *P16*, *LAMAI*, and *CDHI*. These results confirm previous findings for *RASSF1A*, *CTNNB1*, and *P16*.<sup>10,17</sup>

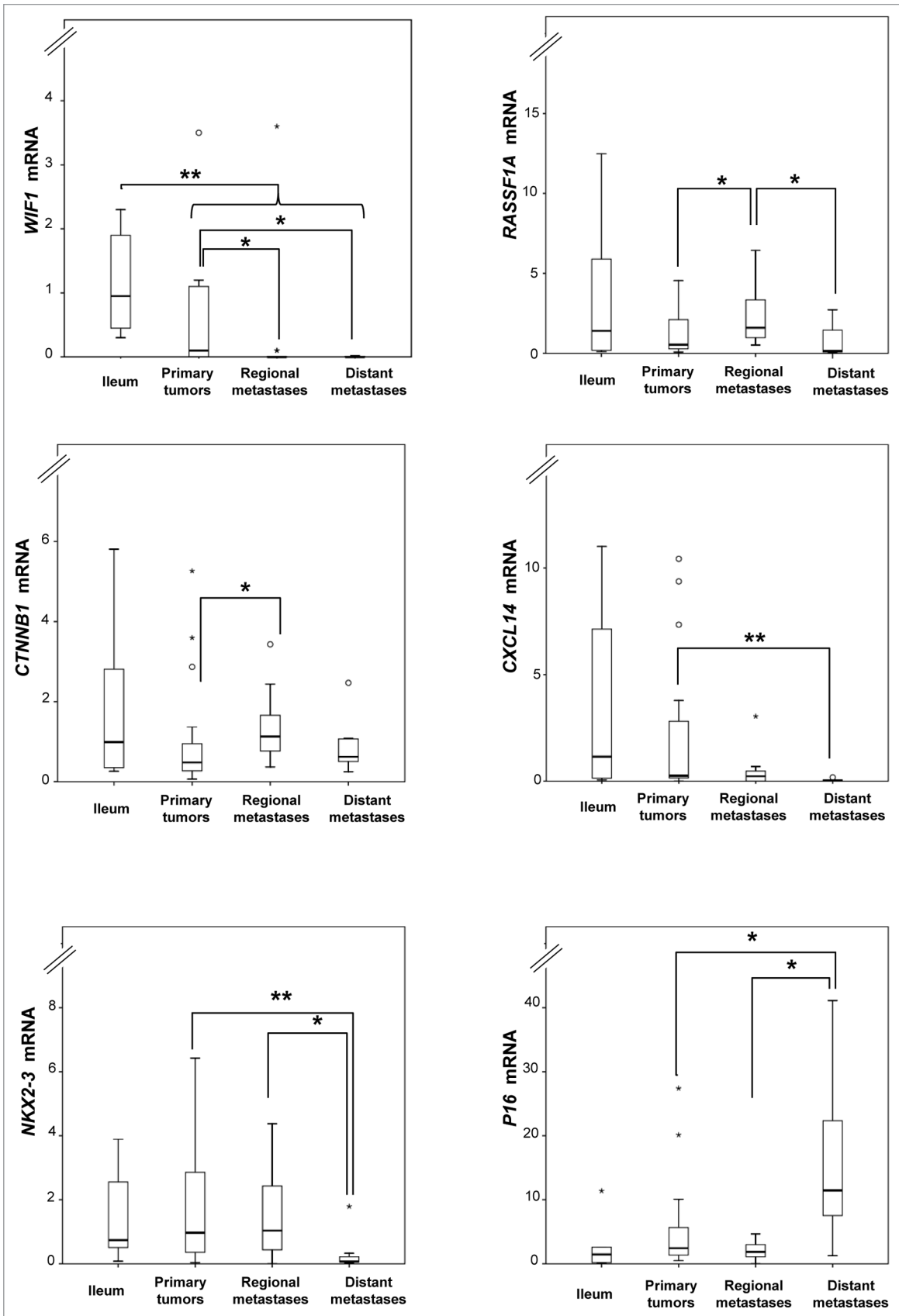
The most heavily methylated gene, *WIFI* (wnt inhibitory factor), was downregulated in metastases compared with primary tumors and ileum. Furthermore, tumors with *WIFI* hypermethylation preferentially fell into Cluster I, which was also

characterized by global hypomethylation and loss in 18q (the most common genomic alteration in SI-NETs). *WIFI* was highly methylated in both CNDT2 (MetI 99%) and HC45 (MetI 38%) cell lines, and treatment with 5-aza-CR reduced methylation density and restored gene expression. *WIFI* is a Wnt antagonist that inhibits the interaction of Wnt with its receptor, named Frizzled. This in turn prevents cytoplasmic accumulation and nuclear translocation of CTNNB1 ( $\beta$ -catenin), which would otherwise activate transcription of oncogenic factors such as c-Myc and TERT, the catalytic subunit of telomerase.<sup>18</sup> Epigenetic silencing of *WIFI* has been reported in squamous cell carcinoma of the cervix,<sup>19</sup> breast cancer,<sup>20</sup> bladder cancer,<sup>21</sup> colorectal cancer,<sup>22</sup> nasopharyngeal and esophageal carcinoma,<sup>23</sup> and non-small-cell lung cancer.<sup>14</sup> In pancreatic and lung carcinoid cell lines absent or reduced *WIFI* expression was observed without accompanying promoter methylation.<sup>24</sup>

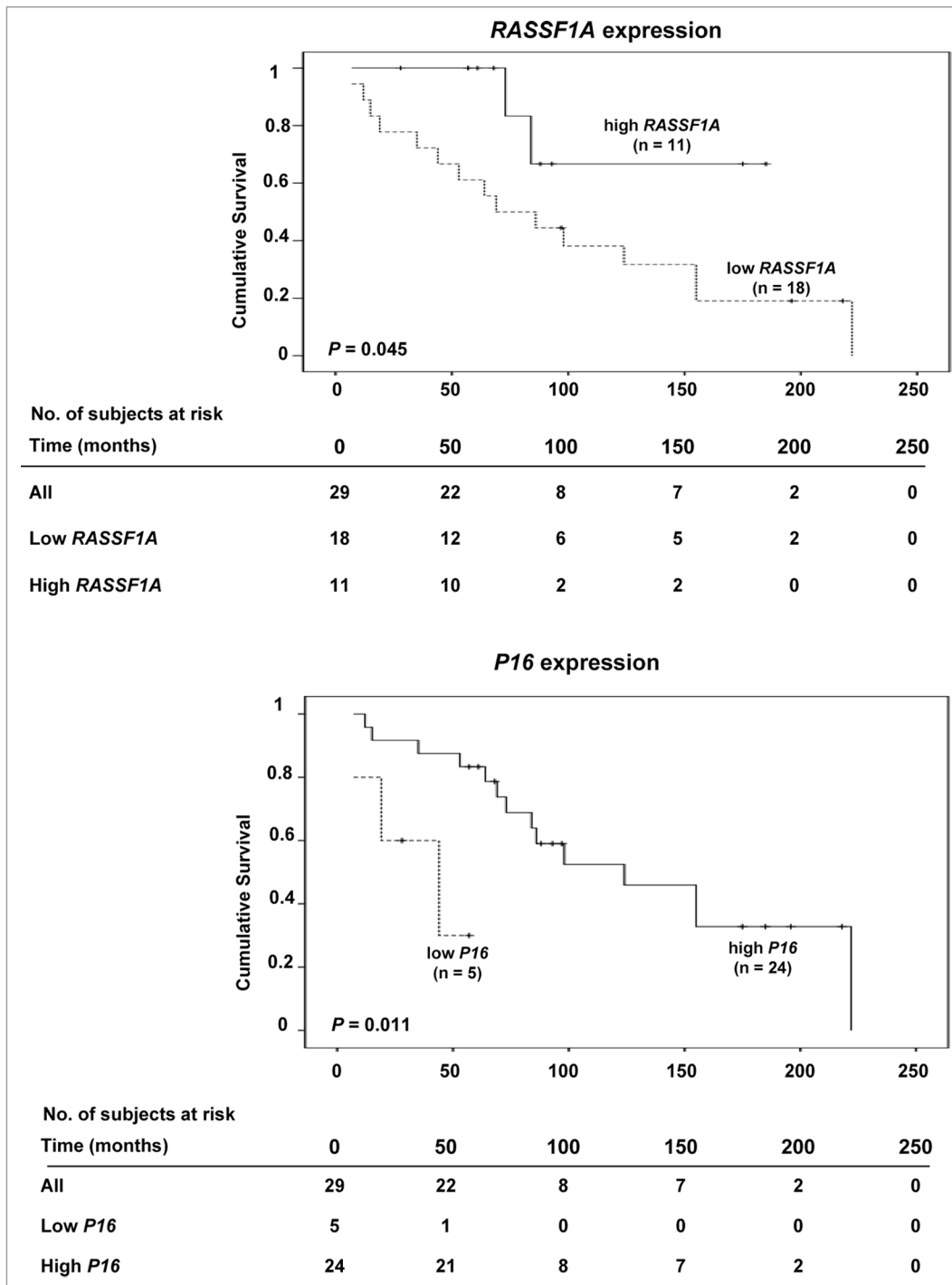
We found increased methylation of *RASSF1A* in distant metastases together with reduced gene expression, in agreement with previous studies.<sup>10,17</sup> Moreover, low *RASSF1A* expression was associated with shorter survival. *RASSF1A* hypermethylation has been shown in different cancer types including SI-NETs.<sup>10</sup> It contributes to cancer development basically through modulation of cyclin D1 accumulation, inhibition of the JNK pathway<sup>25</sup> and pro-apoptotic activities achieved by binding to MST1 (Mammalian Sterile Twenty 1) and other apoptotic agents.<sup>26</sup> *RASSF1A* has a role in the modulation of tubulin dynamics and its localization to centrosomes and mitotic spindles.<sup>27</sup> It also inhibits cell motility and promotes cell-cell adhesion,<sup>28</sup> which is in line with our observation of its epigenetic inactivation in distant metastasis. We also identified higher MetI in SI-NETs from female as compared with male cases. However, we did not observe a similar gender-specific difference between normal blood samples arguing against a constitutional difference. Analysis of larger numbers of matched blood and SI-NETs samples could establish whether the alteration is a tumor-specific event. Furthermore, it may be noted that *RASSF1A* methylation was one of the alterations distinguishing Cluster I, which could be partly attributed to differences in gender distribution between clusters.

High methylation and downregulation was observed for *CTNNB1* in a subset of tumors, in agreement with a previous report.<sup>10</sup> However, this was only observed in a small subset of cases, and never in metastases, suggesting that *CTNNB1* does not have a major role as tumor suppressor in SI-NETs.

Our study confirms a previous report of downregulated mRNA expression for *CXCL14* and *NKX2-3* in progressive SI-NETs,<sup>5</sup> and also proposes an epigenetic background for this observation. The chemokine *CXCL14* is downregulated in prostate cancer cells and restoration of its expression by 5-Aza-2-deoxycytidine increases chemo-attraction for dendritic immune cells.<sup>29</sup> In addition, forced expression of *CXCL14* in lung cancer cells, where it is constitutively silenced by DNA methylation, rendered up to 90% necrosis and tumor shrinkage in xenograft models.<sup>30</sup> Promoter hypermethylation of *NKX2-3*, encoding a homeodomain-containing transcription factor, has been reported in melanoma cell lines.<sup>31</sup> Silencing of *NKX2-3* by siRNA have



**Figure 3.** Relative mRNA expression levels determined by qRT-PCR for selected loci in normal ileum references and tumor subgroups. Fold changes are represented as compared with the mean of ileum (assigned the arbitrary value of 1). *P* values for statistical comparisons between groups are indicated for suggestive *P* values  $\leq 0.05$  (\*) or  $\leq 0.01$  (\*\*).



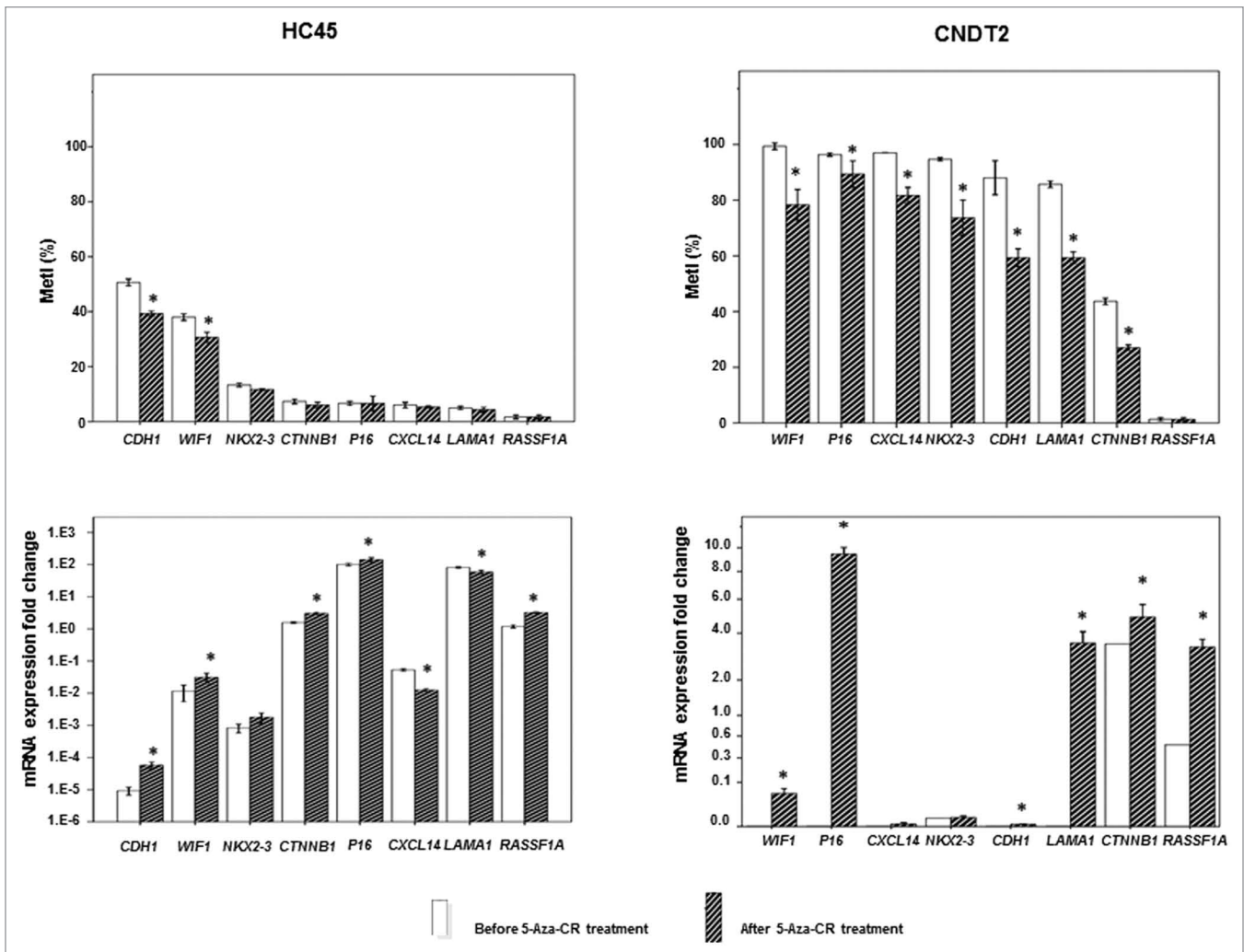
**Figure 4.** Kaplan-Meier survival curves comparing overall survival in SI-NET cases with high or low mRNA expression of *RASSF1A* (top) and *P16* (bottom).

suggested a role for Nkx2–3 in colorectal cancer development by regulation of the Wnt signaling pathway.<sup>32</sup>

The possibility of epigenetic inactivation of *P16* in SI-NETs is controversial.<sup>9,13,17</sup> We detected increased *P16* promoter methylation in a few tumors only, suggesting a limited role of inactivation by promoter methylation. *P16* overexpression has been suggested to keep tumors in “oncogene-induced senescence” and low-proliferative states, and *P16* inhibition may contribute to malignant

transformation.<sup>33,34</sup> The current study supports this hypothesis in that a worse patient outcome was observed for cases with *P16* downregulation. Age at diagnosis was identified as a confounding factor in agreement with observations of altered *P16* in relation to age.<sup>35</sup>

The *CDH1* gene is located in 16q and *LAMA1* in 18p, chromosomal regions that are frequently affected by copy number losses as shown in our previous study of the same samples.<sup>15</sup>



**Figure 5.** Promoter methylation and gene expression in SI-NET cell lines treated by 5µM 5-aza-CR. At the top is shown MetI in HC45 and CNDT2 cell lines treated with 5-aza-CR. Corresponding mRNA expression fold changes are shown below. The values are in relation to SI-NET 30P in which all genes were expressed (assigned the arbitrary value 1.0). Results before and after 5-aza-CR treatments are indicated by white and striped bars, respectively. *P* values for statistical comparisons between groups are indicated for suggestive *P* values ≤ 0.050 (\*).

Epigenetic inactivation by methylation and a reduction in expression would be consistent with bi-allelic inactivation according to Knudson's two-hit hypothesis.<sup>36</sup> However, the methylation density was not found to be higher in tumors than in normal ileum and blood controls for these two genes. Hence, DNA methylation is not expected to be the only mechanism involved. *CDH1* and *LAMA1* MetIs were positively correlated, and low MetIs as well as increased *CDH1* mRNA expression were associated with loss in chromosome 18p. Furthermore, hypomethylation of *CDH3*, located adjacent to its homolog *CDH1*, has previously been observed in cancer.<sup>37</sup>

We found global hypomethylation in SI-NETs and, particularly, in distant metastases, a phenomenon attributed to overriding transposon activity control and genome integrity. Both ELISA and LINE1 pyrosequencing results were valuable indicators of global methylation, acting in a firm pattern of correlations with promoter methylation alterations. While LINE1 analysis directly represents retrotransposon methylation and silent status,

ELISA gives an overall ratio of 5-methyl cytosine over other nucleotides in the genome. Hydroxymethyl cytosines, which were recently implicated in cancer,<sup>38</sup> are not included in the estimations by ELISA but are taken into account by pyrosequencing. Using either method, hypomethylation was detected in a large proportion of samples. Hypomethylation of LINE1 and Alu in a cohort of pancreatic and ileal and non-ileal NETs has previously been reported by Choi et al.<sup>11</sup> In that study, hypomethylation was more common in ileal NETs than in other NETs. Our study confirms the association of SI-NETs with global hypomethylation, *RASSF1A* hypermethylation and chromosome 18 loss. Furthermore, LINE1 methylation was correlated with *CDH1* and *LAMA1* and inversely correlated with *WIF1* MetI. Hypomethylation by ELISA was also correlated with *WIF1* and inversely to *LAMA1* MetI. In addition, distant metastases were hypomethylated compared with the primary tumors.

A demethylation study on two SI-NET cell line models was conducted. Both cell lines were highly methylated for some of the



genes found methylated in tumors. 5-aza-CR treatment reduced methylation density of global LINE1 repeats and of individual promoter CpG islands. The inhibition of promoter methylation was accompanied by restoration of gene expression, suggesting a causative effect of promoter methylation on gene expression levels.

In conclusion, global hypomethylation and promoter methylation was demonstrated in SI-NETs with related effects on gene expression observed in vitro. Continued studies to explore SI-NET development may focus on *WIFI* inactivation by methylation and on *RASSF1A* and *PI6* expression as potential prognostic markers for refined clinical handling of SI-NETs.

## Materials and Methods

### SI-NET and reference samples

A total number of 44 fresh frozen sporadic SI-NETs from 33 patients were obtained from Karolinska University Hospital biobank (Table S3, case 1–33). Twenty-four tumors were primary and 20 were metastasis including 7 distant (5 liver and 2 ovarian) and 13 regional metastases. From 9 patients paired primary tumor and metastasis were available. A detailed description of the clinical and histopathological characteristics of case 1–32 has been published, together with data on copy number alterations.<sup>15</sup> In addition, a regional metastasis from a patient with family history of SI-NET (case 33) was included. Two metastases from a patient with colorectal carcinoid were also included for comparison. All samples were obtained with informed oral consent as documented in the patient's medical record and the collection and study of patient material was approved by the local Ethics Committee. Nine DNA samples from anonymized normal ileum, peripheral blood from 6 of the SI-NET cases, and pooled samples of peripheral blood from 10 female or 10 male healthy individuals (Promega, G1521 and G1471) were used as references.

### Cell lines

The cell lines HC45 and CNDT2 are both of human ileal origin derived from liver metastases. HC45 was a kind gift from Professor RV Lloyd, Mayo Clinic,<sup>39</sup> and CNDT2 was kindly provided by Professor LM Elis, MD Anderson Cancer Center.<sup>40</sup> The authenticity was demonstrated by genotyping of short tandem repeats (STRs) at Biosynthesis Inc. (Table S4).

### Extraction of DNA and RNA

Genomic DNA and RNA were extracted simultaneously from fresh frozen tissues and cell pellets following the manufacturer's instructions (Allprep DNA/RNA/Protein, Qiagen) and measured by Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies).

### Pyrosequencing

500 ng genomic DNA from each sample was bisulfite converted following the protocol of the manufacturer (Epitect Bisulfite, Qiagen, or EZ DNA methylation kit, Zymo Research) and quantified with Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies) to apply 17 ng in the PCR amplification (Pyromark PCR, Qiagen). PCR conditions were 95 °C activation step for 15 min, followed by 40–45 cycles of amplification

(denaturation at 94 °C, annealing at 50 °C, 55 °C, or 56 °C, and extension at 72 °C, each for 30 s) and a final extension of 72 °C for 10 min. Annealing temperature, concentration of MgCl<sub>2</sub> and quality of PCR product were optimized and assessed for each assay by electrophoresis in 1.5% agarose gels. Commercially available assays (Table S5) were used together with a PyroMark Q24 instrument and PyroMark Gold Q24 Reagents according to the manufacturer's instructions (Qiagen). Controls were run for each assay including: 0, 50 and 100% methylated DNA (Qiagen) to confirm the fidelity of Pyrogram peaks; a PCR without template DNA to confirm lack of interaction between primers and contamination; a PCR with template DNA without sequencing primer to confirm lack of template looping background; and reaction with sequencing primer without template to confirm lack of sequencing primer hairpin background. Data were analyzed using the PyroMark Q24 software. For each gene and sample a mean methylation density was calculated based on the values for the individual CpGs assayed. Samples with MetI ≤ 10% were regarded as having a low methylation of experimental and biological low significance. Samples with MetI > 10% were classified as methylated. Samples were assigned as hypermethylated if the MetI was above the mean for the entire group of normal ileal samples. Thus the cut-off for methylation was 10% or higher. The following genes were studied: *WIFI*, *RASSF1A*, *CTNNB1*, *CXCL14*, *NKX2-3*, *PI6*, *LAMA1*, *CDH1*, *CDH3*, *PI4*, *SMAD2*, *SMAD4*, *HIC1*, and *APC* (Table S5).

LINE1 was similarly analyzed by Pyrosequencing as an indicator of global DNA methylation. SI-NETs with a MetI below the mean of normal ileum were regarded as hypomethylated.

### ELISA-based quantification of global DNA methylation

Antibody against 5-methyl cytosine was applied in an ELISA-based global DNA methylation quantification method following the manufacturer's instructions (MethylFlash™ Methylated DNA Quantification, Colorimetric, Epigentek). Briefly, DNA was bound to the strip wells with high DNA affinity and was incubated with capture and detection antibodies. The absorbance was read at 450 nm using a microplate reader (VERSAmax; Molecular Devices). The absolute quantity of 5-methyl cytosine in the global DNA was determined by subtracting the sample OD from the negative control divided by the slope of a standard curve, which was obtained from positive control DNA with known 5-methyl cytosine content, running on the same plate in duplicate.

### Real Time Quantitative PCR (qRT-PCR)

Reverse transcription was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). 100 ng RNA was used as described before.<sup>41</sup> The quality and concentration of cDNA was assessed by spectrophotometry. 100–1500 ng of cDNA per reaction was applied to the TaqMan expression assays (Table S5) following the manufacturer's instructions (Applied Biosystems). *ACTB* (Beta-actin) was run as endogenous control in the same plate for each assay. Each sample was run in triplicate and non-template control was included in each plate. Plates were run using Applied Biosystems 7900 instrument and analyzed with the SDS 2.4 software. Mean C<sub>T</sub> values were normalized against *ACTB* to calculate ΔC<sub>T</sub> for each sample and fold

changes were calculated by the  $2^{-\Delta\Delta CT}$  method, as  $\Delta\Delta C_T = \Delta C_T$  (sample) -  $\Delta C_T$  (mean of normal ileum).<sup>42</sup>

#### In vitro demethylation

Each cell line was cultured in DMEM, with L-glutamine and high glucose supplemented with 10% FBS (Invitrogen, 41965039 and 10500056) in T25 flasks and was maintained at 37 °C, humidified incubator with 5% CO<sub>2</sub> in triplicate. After 24 h the cells were at 40% confluency and 5-aza-CR (Sigma Aldrich, A2385) treatment was started for 4 consecutive days. The medium was replaced every day by freshly prepared 0 or 5 μM 5-aza-CR. The concentration of drug was optimized beforehand, by ELISA in 0, 10 and 20 nM 5-aza-CR and refined using LINE1 assay in a range of 0 to 10 μM. Accordingly, a concentration of 5 μM 5-aza-CR was used in the further experiments. Doubling time was already measured as 2 days and accordingly in 4 days 75% of DNA molecules were expected to be unmethylated. The cell pellet harvested for extraction by Trypsin/PBS (1%, Invitrogen, 15400054) after washing with PBS. DNA and RNA extracted from treated and untreated cells were analyzed by Pyrosequencing and qRT-PCR.

#### Statistical and clustering analysis

The Mann Whitney *U* test was used to compare mean values between all double groups of continuous variables and Chi-square to compare mean values of every two or several categorical groups. Log-rank test was used to evaluate the effect of each variable on the survival and illustrated by Kaplan-Meier plots. Multivariate Cox regression was used to evaluate cofounders involvement in survival analysis and multiple regressions to evaluate the hypothesis of tumor stage being cofounding factor in *CTBNI* overexpression correlation with tumor size. For paired

samples only laboratory data of the metastasis were considered for survival analysis and comparison to clinical characteristics. All statistical analyses were performed using the statistical software SPSS v 16.0. Observed differences with *P* values ≤ 0.050 were reported. Given the large number of comparisons performed, a conservative approach was taken in the interpretation of statistically significant differences. *P* values ≤ 0.001 were reported as statistically significant and *P* values ≤ 0.050 were regarded as being of suggestive significance.

Methylation densities of the methylated genes in tumor samples were standardized in a range of 0 to 1 and subjected to unsupervised hierarchical clustering, Euclidian method using MeV<sup>43</sup>.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Funding

This work was supported by grants from the Swedish Cancer Society, the Swedish Research Council, the Gustav V Jubilee Foundation, the Cancer Society in Stockholm, Karolinska Institutet, and Stockholm County Council.

#### Acknowledgments

The authors wish to thank Ms. Lisa Ånfalk, Karolinska University Hospital for excellent assistance in tissue collection and the medical genetics group for valuable discussions.

#### Supplemental Materials

Supplemental materials may be found here: [www.landesbioscience.com/journals/epigenetics/article/28936](http://www.landesbioscience.com/journals/epigenetics/article/28936)

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