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RESEARCH PAPER

Telomerase activation in small intestinal neuroendocrine tumours is associated with aberrant *TERT* promoter methylation, but not hot-spot mutations

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ABSTRACT

Telomere maintenance is a critical requirement for enabling replicative immortality and tumour development. Here, telomerase expression and activity, telomere length (TL) and potential regulatory factors that can underlie telomerase machinery alterations in small intestinal neuroendocrine tumours (SI-NETs) were analyzed. Telomerase activity assessed by TRAP assay was increased in SI-NETs compared to normal ileum ($P < 0.001$). The telomerase reverse transcriptase gene (*TERT*) was over-expressed in SI-NETs vs. normal ileal samples ($P = 0.01$). Furthermore, relative TL assessed by qPCR was found shorter in tumours compared with normal ileum ($P = 0.02$) and in distant metastasis samples compared to primary tumours and local metastases ($P= 0.02$). *TERT* promoter hotspot mutations were not present and *TERT* copy number gain was only observed in 3/70 tumour samples. *TERT* or chromosome 18 copy number alterations were not associated with telomerase expression and activity or TL. However, hypermethylation of *TERT* promoter in Region B – in the proximity of the transcription start site – was inversely correlated with *TERT* expression and telomerase activity and positively correlated with TL. Global LINE1 methylation was positively correlated with *TERT* promoter Region B methylation and was inversely correlated with telomerase activity, *TERT* expression and the upstream Region A methylation. The results show that telomerase activation, *TERT* expression and shorter telomeres are commonly found in SI-NETs. Aberrant DNA methylation of *TERT* promoter and of LINE1 can be implicated in abnormal regulation of *TERT* in SI-NETs.

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Introduction

Small intestinal neuroendocrine tumours (SI-NETs) are slow-growing endocrine malignancies that are often metastasized at the initial diagnosis, thus often causing lifelong symptoms [1]. Due to the long-lasting nature of these tumours, enabling replicative immortality could potentially be considered as a major driver in tumour development of SI-NETs.

Telomere erosion and aberrant telomerase activity are considered as important factors in the development of many tumours including endocrine tumours [2]. The Hayflick limit leads to senescence after multiple cell divisions unless the emerging malignant cells bypass telomere shortening through aberrant activation of telomere extension mechanisms [3]. While normally absent in non-immortalized cells, telomerase activity has been detected in around 90% of human cancer cells [4].

Telomerase activation has been linked to many endocrine malignancies [2]. Telomerase activity and *TERT* over-expression have been associated with *TERT* promoter mutation and DNA copy number gains in follicular cell-derived thyroid carcinoma and with *TERT* DNA methylation at an upstream promoter region in medullary thyroid carcinoma [5]. While increased methylation at an upstream region (Region A at positions −578 to −541) was associated with poor outcome [6,7], low methylation of a downstream region (region B at −162 to −100 bp) close to the transcription start site (TSS) was associated with *TERT* expression *in vitro* [8] as well as in adrenocortical carcinomas [9]. Furthermore, in pancreatic neuroendocrine tumours the *ATRX* and *DAXX* genes are

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recurrently mutated [10], a phenomenon that is strongly associated with the 'alternative lengthening of telomeres' (ALT) pathway [11], however, these genes have not been found recurrently mutated in SI-NETs [12].

Still, our knowledge on telomeres and telomerase activity in SI-NETs is limited. In this study, we detected telomerase activity, *TERT* expression and telomere length alteration in SI-NETs and investigated the possible underlying mechanisms, including *TERT* copy number alteration, as well as *TERT* promoter methylation density and mutational status.

Materials and methods

Tissue samples

Frozen tissue samples of SI-NETs and normal adjacent ileum were obtained from the Karolinska University Hospital Biobank. Samples were collected with informed patient consent and an ethical committee approved the study. The collection of tissue samples had been done according to a standardized procedure whereby samples were snap-frozen in liquid nitrogen and stored at −86°C for up to 33 y until use. A total of 70 tumour samples from 50 patients were studied including 37 primary tumours, 23 regional metastases and 10 distant metastases (Supplementary Table S1). Tumour characteristics of 44 tumours from 32 patients were reported in our previous study [13]. An extended number of 26 samples from 18 patients were also included in this study. Fifteen ileal adjacent tissue samples were used for comparison to a non-tumour tissue.

Assessment of telomerase activity

Telomerase activity was assessed for 49 SI-NETs and 3 normal ileal samples in duplicate using TeloTAGGG Telomerase PCR ELISA kit (Roche Diagnostics GmbH) following the manufacturer's instructions. HEK-293 lysate provided with the kit served as a positive control and its heat-inactivated counterpart as a negative control. The absorbance was read at OD450-OD670. OD values were subtracted from lysis buffer background absorbance and were normalized to positive control. Values >0.2 were considered as positive for telomerase activity.

DNA and RNA extraction

DNA and RNA were extracted from fresh frozen tissues using Qiagen using DNeasy Blood and tissue and RNeasy Plus Mini kit, following the manufacturer's instructions (Qiagen).

TERT expression analyses

TERT expression was assessed for 63 SI-NETs and 12 normal ileal samples using TaqMan assay Hs00972656_m1 (Thermo Fisher Scientific) based on the previously described delta-delta Ct method [14]. cDNA synthesis was performed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) and qPCR was performed in an 7900HT Applied Biosystems instrument. Ct values were normalized to *ACTB* Hs99999903_m1 (Thermo Fisher Scientific).

TERT mutation analyses

A quantity of 50 ng genomic DNA for 70 SI-NETs was amplified using AmpliTaq Gold® 360 Master Mix and PCR primers for Sanger sequencing analysis of the C228T and C250T hotspot mutation region in the *TERT* promoter, using primers F: 5′-CACCCGTCCTGCCCCTTCACCTT-3′ and R: 5′-GGCTTCCCACGTGCGCAGCAGGA-3′.

DNA bisulfite conversion and pyrosequencing

One hundred to 500 ng DNA from 70 SI-NETs was bisulfite converted using EpiTect Bisulfit kit (Qiagen). Twenty ng bisulfite converted DNA was amplified in two different regions of the *TERT* promoter. The upstream methylation Region A, encompassing positions **−5**78 to −541 was amplified using biotinylated primer-F: 5′- GGGTTTGTGTTAAGGAGTTTAA GT-3′ and primer-R: 5′- AAACCCAAAACTACCT CCA-3′. The downstream methylation Region B, encompassing positions −162 to −100 bp was targeted using primer-F: 5′- GGTGGTAGGGGTTAGGGTT TTTTA-3′ and biotinylated primer-R: 5′- TACCCC TTCACCTTCCAACTC-3′. The following sequence was analyzed for Region A: 5′-**CG**GCATT **CG**TGGTGCC**CG**GAGCC**CG**A**CG**CCC**CGCG**TC-**CG**G-3′ (CpG sites in bold). The sequence analyzed

for Region B was: 5′-CCC**CG**TCC**CG**ACCC CTCC**CG**GGTCCC**CG**GCCCAGCCCCCTC**CG**GG CCCTCC CAGCCCCTCCCC-3′ (where CpGs are specified in bold, C250T and C228T mutation hotspots indicated in simple and double underline, respectively).

Pyrosequencing was performed with previously described methodology [14] using sequencing primer-S: 5′- CCAAAACTACCTCCAAAT-3′ for Region A and primer-S: 5′- GGGGTAGAGG AAAGGAA-3′ for Region B. LINE1 methylation was assessed for 70 SI-NETs and 15 normal ileal samples by Pyrosequencing methodology described before [14]. For LINE1 results have been previously published for a subset of cases (Supplementary Table S1 [14];). We identified Methylation Index (MetI) as the mean of methylation density for all representative CpG sites in one examined region.

TERT and CDH19 copy number analysis

DNA copy numbers of *TERT* were determined in 70 SI-NETs using TaqMan Copy Number Assay (Thermo Fisher Scientific, Hs01237576_cn) and an endogenous control assay, *RNaseP* (Thermo Fisher Scientific, part number 4403326). The values were calibrated to a commercially available pool of 10 healthy individual blood DNA samples (Promega). The reactions were run in quadruplicate in a Step One Plus qRT-PCR machine following the manufacturer's instructions (Thermo Fisher Scientific) [15]. We also assessed chromosome 18q copy numbers using *CDH19* TaqMan copy number assay (Thermo Fisher Scientific, Hs02826809_cn), and *RNase P* as a reference gene, as explained in detail before [13]. For a subset of cases results for *CDH19* have been previously published [13].

Assessment of telomere length

Relative telomere length was determined using previously described methodology [16,17]. Briefly, 5 ng DNA was amplified for 70 SI-NETs and 13 normal ileal samples in duplicate in a CYBR green qPCR reaction (Thermo Fisher Scientific) using primers against telomeres: (Tel 1b 5′-CGGTTTGTTTGGGTT TGGGTTTGGGTTTGGGTTTGGGTT-3′ and Tel 2b 5′-GGCTTGCCTTACCCTTACCCTTACCCTT ACCCTTACCCT-3′). The mean Ct values were normalized (dCt) to the endogenous control *HGB*, F: 5′-TGTGCTGGCCCATCACTTTG-3′ and R: 5′-ACC AGCCACCACTTTCTGATAGG-3′. Pooled healthy blood DNA samples (Promega) were run as a reference to calculate ddCt = dCt- mean reference Ct. Telomere length was then calculated as 2^-ddCt.

Statistical analysis

Categorical and continuous variables were analyzed using Fisher Exact test and Mann Whitney U test, respectively. For correlation analysis, Spearman rank order correlation was used. IBM SPSS Statistics 22 was used for all statistical analyses. P-values <0.05 were defined as statistically significant.

Results

Telomerase activation and increased TERT expression in SI-NETs

Telomerase activity was detected in 27/49 of tumour samples (55%, range 0.08–0.88 normalized to the positive control, HEK293 cell lysate), but in none of three normal ileal tissue samples used as non-tumour comparison. There was a significantly higher telomerase activity in tumours compared to non-tumour samples ($P< 0.001$, Figure 1(a)).

TERT expression was determined by quantitative PCR and found detectable in 60/63 (95%) SI-NETs and in 8/12 (67%) normal ileum samples. Lower levels were observed in normal ileum (median 0.14) as compared to SI-NETs (median 3.29) ($P= 0.01$, Figure 1(b) Supplementary Table S1).

Shorter telomere lengths in SI-NETs

Relative telomere length was assessed by a qPCR-based method. Telomeres were found shorter in tumours compared with normal ileal samples ($P= 0.02$, Figure 1(c)). Furthermore, comparison of tumour subgroups revealed shorter telomeres in distant metastasis as compared to primary tumours and regional metastases ($P= 0.02$, Figure 1(c)).

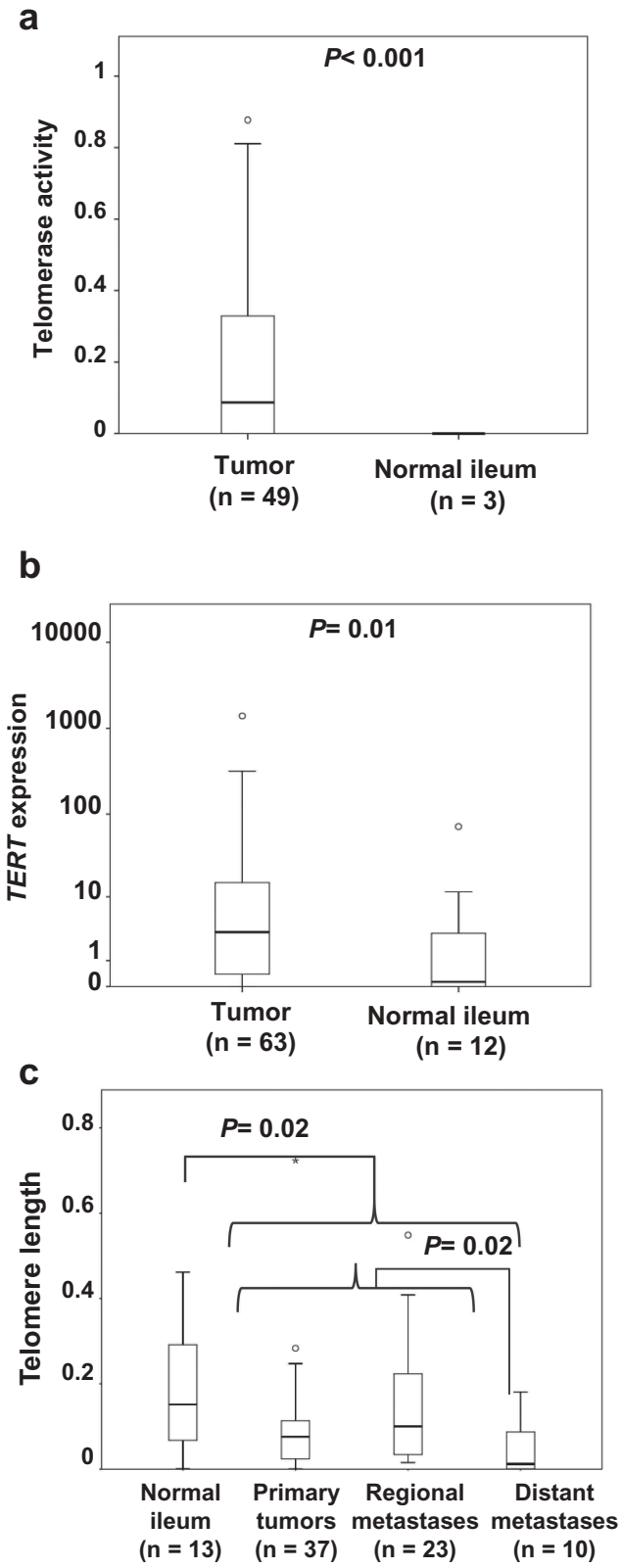

Figure 1. Aberrant telomerase activity, *TERT* expression and telomere length in SI-NETs. (a) Telomerase activity in normal ileum compared with SI-NETs. (b) *TERT* expression in normal ileum compared with SI-NETs. (c) Telomere length in normal ileum and different subgroups of SI-NETs.

Low frequency of TERT promoter mutations and TERT copy number gain in SI-NETs

The *TERT* promoter region spanning the two reported hotspot mutations known as C228T and C250T was sequenced using Sanger sequencing. No mutation was detected in any of the 70 tumour samples analyzed, suggesting that *TERT* promoter mutations are rare in the SI-NET entity.

TERT copy number was assessed using TaqMan copy number assay in all 70 SI-NETs and 13 normal ileum samples. Copy number gain was observed in 3 SI-NETs, including two with gain from 2 to 3 copies and one case with amplification to 10 copies (Supplementary Table S1). All normal ileal samples were found diploid. *TERT* copy number alterations were not found to be associated with telomerase activation, *TERT* expression or telomere length.

Aberrant TERT promoter methylation in SI-NETs

We quantified DNA methylation at two different regions on *TERT* promoter, a downstream *TERT* promoter region (Region B) spanning the C228T and C250T hotspot mutation sites, including 5 CpG sites and an upstream *TERT* promoter region (Region A) including 8 CpG sites (Figure 2(a)). For Region B the methylation index (MetI) ranged from 2% to 32% in the 68 SI-NET samples and from 4% to 17% in the 11 ileal samples. In Region A, the 70 SI-NETs showed MetI from 9% to 89%, and in the 11 normal ileum samples from 9% to 47%. Some variation in methylation density between CpG sites assessed was noted (Figure 2(a)). For Region B, the MetI was inversely correlated with telomerase activity (P= 0.055, R = −0.279; Table 1 and Figure 2(b)) and inversely correlated with *TERT* expression (P= 0.001, R = −0.414; Table 1 and Figure 2(c)). Telomere length was correlated with MetI at region B (P = 0.015, R = 0.295, Table 1 and Figure 2(d)).

LINE1 methylation

We also analyzed LINE1 methylation as an indicator of global methylation density and possible regulator of *TERT*. LINE1 was found hypomethylated in SI-NETs (range 44–76%) compared to normal ileum (range 71–78%) (P< 0.001) and in distant metastases compared to primary tumours and regional metastases (P= 0.04, Supplementary Figure S1). LINE1 MetI was inversely correlated with telomerase activity (P = 0.003, R = −0.411, Figure 3(a)) and with *TERT* expression (P= 0.003, R = −0.372, Figure 3(b)). LINE1 MetI was correlated with MetI at Region B (P< 0.001, R = 0.566, Figure 3(c)), and inversely correlated with MetI at Region A (P= 0.002, R = −0.357, Figure 3(d)).

Copy number loss at chromosome 18q

We also examined a link between chromosome 18q copy number loss, a hallmark of SI-NETs, and telomere-associated features. Totally 39 tumour samples exhibited copy number loss at 18q while 27 tumours were diploid, and another 3 tumours showed gain with 3 or 4 copies of chromosome 18q. We did not observe any link between chromosome 18 copy number loss at the *CDH19* locus and telomerase activity, *TERT* expression or telomere length (data not shown). However, higher MetI in Region B was observed in SI-NETs with copy number loss of chromosome 18q as compared to tumours without this abnormality (P= 0.03, Supplementary Figure S2).

Discussion

Biological mechanisms underlying development and progression of SI-NETs are still to a large extent unknown. Although telomerase machinery abnormalities have been implicated in many tumours, comprehensive and informative telomere-related studies of SI-NETs are missing. The telomerase complex is now considered as a therapeutic target in various tumours, through different strategies including small molecule inhibitors, antisense oligonucleotides, immunotherapy and gene therapy, some having entered clinical trials phase I or II [18].

In this study, we analyzed alterations in telomerase activity, mRNA expression of its catalytic component *TERT* and telomere length in SI-NETs and sought for possible mechanisms that can underlie those alterations. We found telomerase activity in 55% of analysed tumours but none in normal adjacent ileal controls. *TERT* was over-expressed in tumours as compared to normal ileal samples. A large number of studies indicate the implication of *TERT* and telomerase in

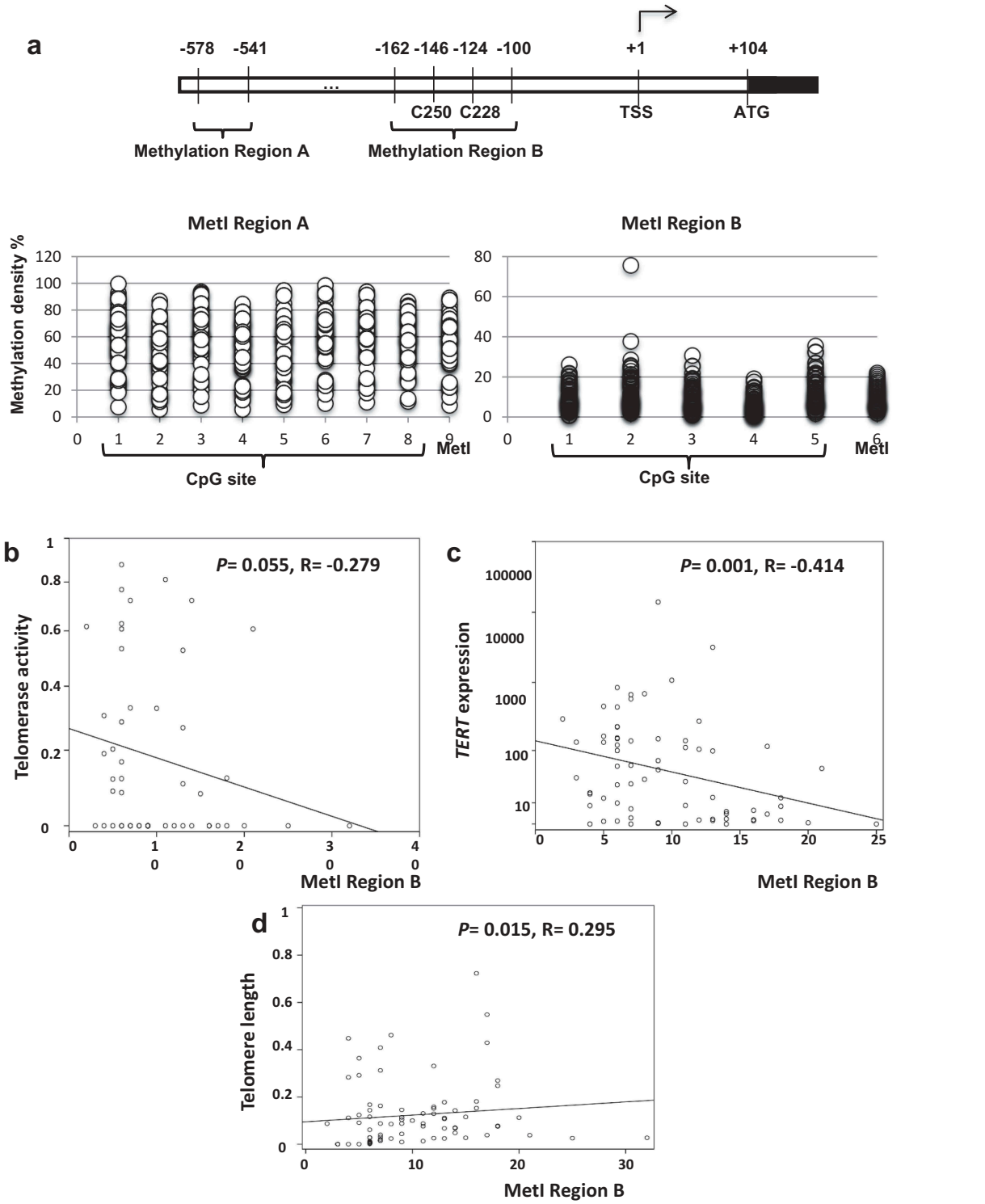

Figure 2. DNA methylation Index (MetI) at *TERT* promoter methylationRegion B. (a) Schematic illustration of the *TERT* promoter with indication of methylation Region A and B, the location of hotspot *TERT* mutations at C250 and C228, and the transcriptional start site (TSS). Below is shown the methylation density determined at 8 CpG sites in Region A and 5 CpG sites in Region B and their mean values (MetI). (b) Inverse correlation between Region B MetI and telomerase activity in SI-NETs. (c) Inverse correlation between Region B MetI and *TERT* expression in SI-NETs. (d) Correlation between Region B MetI and telomere length in SI-NETs.

Table 1. Summary of results from correlation analyses in SI-NETs.

Parameter		Telomerase activity	*TERT* expression	*TERT* MetI Region B	*TERT* MetI Region A	LINE1 MetI	Telomere length
Telomerase activity	Correlation coefficient	1.000	.000	−.279	.090	−.411	−.205
	Sig. (2-tailed)		.999	.055	.537	.003	.157
	N	49	45	48	49	49	49
TERT expression	Correlation coefficient	.000	1.000	−.414	.189	−.372	−.124
	Sig. (2-tailed)	.999		.001	.137	.003	.334
	N	45	63	61	63	63	63
TERT MetI Region B	Correlation coefficient	−.279	−.414	1.000	.108	.566	.295
	Sig. (2-tailed)	.055	.001		.380	.000	.015
	N	48	61	68	68	68	68
TERT MetI Region A	Correlation coefficient	.090	.189	.108	1.000	−.357	−.116
	Sig. (2-tailed)	.537	.137	.380		.002	.338
	N	49	63	68	70	70	70
LINE1 MetI	Correlation coefficient	−.411	−.372	.566	−.357	1.000	.199
	Sig. (2-tailed)	.003	.003	.000	.002		.098
	N	49	63	68	70	70	70
Telomere length	Correlation coefficient	−.205	−.124	.295	−.116	.199	1.000
	Sig. (2-tailed)	.157	.334	.015	.338	.098	
	N	49	63	68	70	70	70

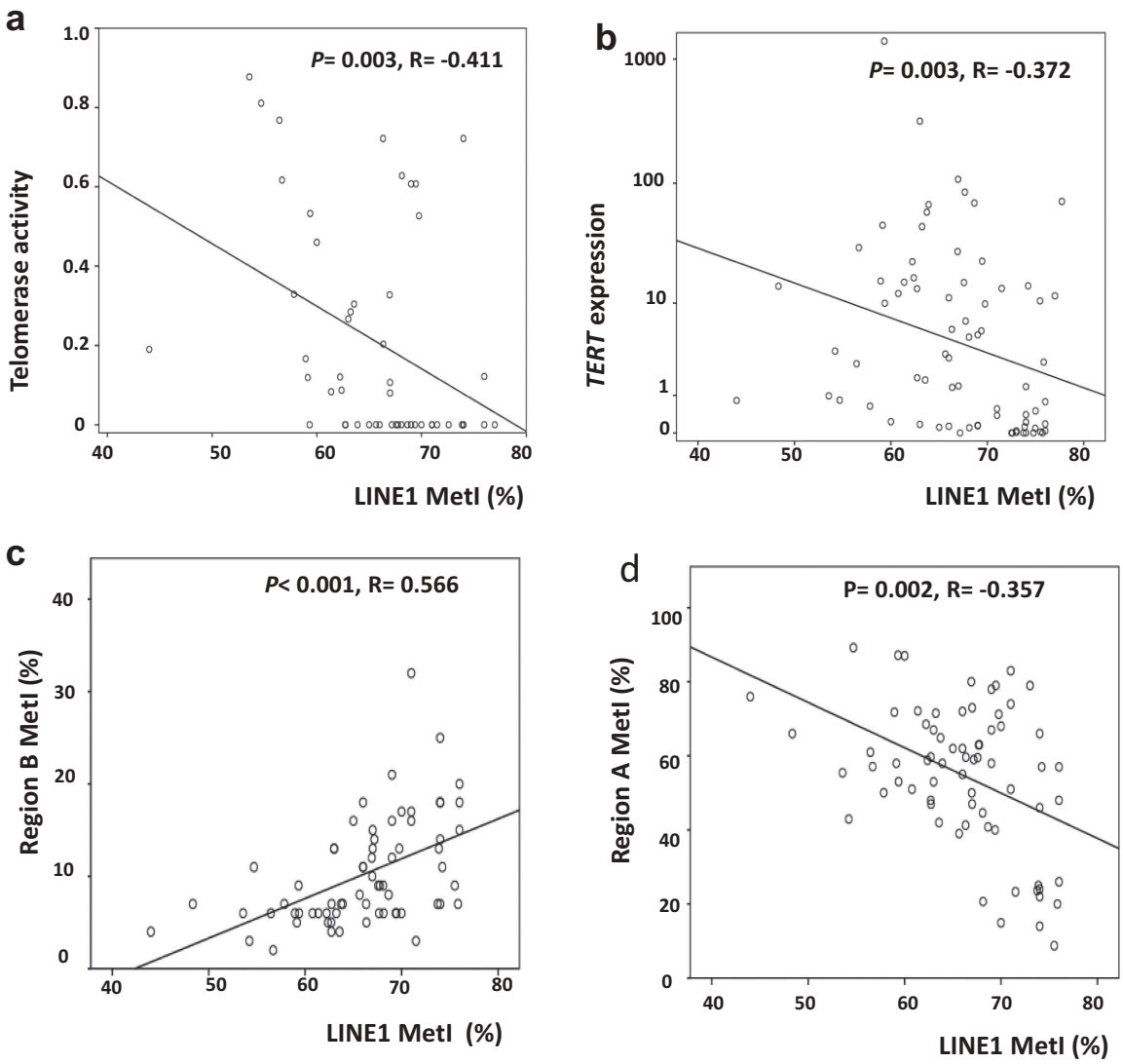

Figure 3. Aberrant LINE1 methylation in SI-NETs. (a) Inverse correlation between LINE1 MetI and telomerase activity. (b) Inverse correlation between LINE1 MetI and *TERT* expression. (c) Correlation between LINE1 MetI and Region B MetI. D. Inverse correlation between LINE1 MetI and Region A MetI.

tumorigeneses. The observed telomerase activity and *TERT* over-expression in SI-NETs demonstrate the involvement of telomerase also in this tumour entity. In this study we used adjacent ileum for comparison of SI-NETs to a non-tumorous tissue, however, the low proportion of enterochromaffin cells from which SI-NETs originate makes this an imperfect reference which is a known dilemma for this disease. With the development of TERT antibodies, a direct comparison of SI-NET cells to normal enterochromaffin cells could be performed by double TERT/Chromogranin A immunohistochemistry, directly on the slides. However, at present, available TERT antibodies are a poor predictor of TERT activation in other tumour types [19].

Telomerase activity is considered a risk factor in many different tumours including pulmonary NETs [20]. However, a previous report on NETs did not find any association between telomerase activity and clinicopathological characteristics [21]. We detected shorter telomeres in distant metastases vs. primary tumours and local metastases, as well as normal ileal samples. Telomere erosion and shorter telomeres in the tumour cells with higher ratio of proliferation especially in metastasized ones are expected [2]. In SI-NETs telomerase activity is enhanced from normal ileum to primary tumours and metastases. Concomitantly, telomeres become shorter, probably due to multiple cell divisions and possibly higher proliferation rate. This could explain a high ratio of chromosomal instabilities; a hallmark of SI-NETs [22], caused by gain of telomerase activity in abnormally eroded chromosomal telomeres.

We also studied the possible mechanisms underlying *TERT* overexpression and the telomerase machinery alterations in SI-NETs. We did not detect any *TERT* promoter mutation in the two hotspots C228T and C250T that can be implicated in many tumours [17]. This is consistent with a recent report indicating rare incidence of these mutations in pancreatic NETs [23]. Moreover, significant copy number alterations of the *TERT* locus on chromosome 5p were also lacking. However, we did see an inverse correlation between higher MetI in Region B and telomerase activation and *TERT* expression and an inverse correlation to shorter telomere length.

The *TERT* promoter methylation analysis illustrated its implication in gene expression and consequently telomerase activity and telomere shortening. The *TERT* promoter is regulated by a variety of transcription factors and regulators [24]. Therefore, aberrant DNA methylation on different promoter loci leads to different effects on *TERT* expression. DNA methylation of the two regions of *TERT* promoter that were analysed in this study has already been reported to have opposite effects on *TERT* expression, as methylation of the transcription start site proximal locus, Region B suppresses *TERT* transcription, while methylation of the distal locus, Region A can pose a transcriptional stimulatory effect [6,25]. We found that Region B MetI was inversely correlated with *TERT* expression and telomerase activity, indicating a typical inhibitory effect posed by promoter CpG methylation on transcriptional machinery [26]. Methylation of this region was also correlated with telomere length.

The loss of chromosome 18q with regards to telomerase machinery alterations was further studied and an association between Region B MetI and chromosome 18q loss was identified; however, we did not find further association between telomere machinery alterations and chromosome 18 loss. Interestingly, Karpathakis and colleagues described clusters with molecular subgroups according to, e.g., CpG hypermethylator phenotype and CNA at chromosome 18 [27].

We analyzed LINE1 methylation in an extended cohort to our previous study [14] and observed LINE1 hypomethylation in distant metastases compared to primary tumours and regional metastases. These observations are in agreement with findings based on global DNA methylation profiling showing global hypomethylation in SI-NETs, which was also more pronounced in liver metastases as compared to primary tumours [28], as well as in regional metastases as compared to primary tumours [29]. LINE1 MetI was inversely correlated with *TERT* expression and telomerase activity. Taking into consideration that LINE1 methylation can inhibit LINE1 expression, this observation is in line with reports that LINE1 induces *TERT* expression and function [30]. Positive correlation between Region B MetI and LINE1 MetI and on the other hand negative correlation between Region A MetI and LINE1 are consistent with the previously reported opposite regulatory functions of these regions [25].

In conclusion, this is the first study that couples aberrant *TERT* promoter methylation to *TERT*

induction in SI-NETs. Our study showed that telomerase activation and *TERT* expression are common in SI-NETs. The C228T and C250T hotspot *TERT* promoter mutations were not present in SI-NETs. However, *TERT* promoter methylation of this mutation-hotspot region may regulate *TERT* expression.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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