TERT promoter hypermethylation is associated with poor prognosis in adrenocortical carcinoma

FREDRIKA SVAHN[1], JOHAN O. PAULSSON[1], ADAM STENMAN[1], OMID FOTOUHI[1], NINNI MU[1], TIMOTHY D. MURTHA[2], REJU KORAH[2], TOBIAS CARLING[2], MARTIN BÄCKDAHL[3], NA WANG[1], C. CHRISTOFER JUHLIN[1] and CATHARINA LARSSON[1]

[1]Department of Oncology-Pathology, Karolinska Institutet, Karolinska University Hospital, Cancer Center Karolinska, SE-17176 Stockholm, Sweden; [2]Department of Surgery, Yale School of Medicine, Yale Endocrine Neoplasia Laboratory, Yale School of Medicine, New Haven, CT 06520, USA; [3]Department of Molecular Medicine and Surgery, Karolinska Institutet, Karolinska University Hospital, SE-17176 Stockholm, Sweden

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Abstract. Telomere maintenance, most commonly achieved by telomerase activation through induction of the telomerase reverse transcriptase (*TERT*) gene, is required for cell immortalization, a hallmark of cancer. Adrenocortical carcinoma (ACC) is an endocrine tumor for which *TERT* promoter mutations and telomerase activation have been reported. The present study assessed alterations of the *TERT* gene locus and telomere length in relation to clinical characteristics in ACC. In total, 38 cases of ACC with known *TERT* promoter mutational status were included. *TERT* promoter methylation densities were assessed by pyrosequencing, and *TERT* copy numbers and telomere length were determined by quantitative polymerase chain reaction analysis, followed by comparison of the mRNA expression of *TERT* and clinical parameters. The ACC tissue samples showed increased *TERT* copy numbers, compared with normal adrenal tissue (NAT) samples (P=0.001). Mutually exclusive *TERT* copy number gains or promoter mutation were present in 70% of the ACC samples. The ACC tissues exhibited higher levels of CpG promoter methylation of all eight CpG sites investigated within the -578 to -541 bp (Region A), compared with the NATs (P=0.001). High methylation density at this region was associated with metastatic disease and/or relapse, poor survival rates and higher European Network for the Study of Adrenal Tumor stage (P<0.05). The mRNA expression of *TERT* was inversely correlated with methylation density at -162 to -100 bp (Region B). Correlation was observed between relative telomere length and the gene expression of *TERT*. It was concluded that epigenetic alterations of the *TERT* promoter are frequent and associated with advanced disease and poorer clinical outcome in ACC.

Correspondence to: Dr Fredrika Svahn or Dr Adam Stenman, Department of Oncology-Pathology, Karolinska Institutet, Karolinska University Hospital, Cancer Center Karolinska, Solna R8:04, SE-17176 Stockholm, Sweden
E-mail: fredrika.svahn@ki.se
E-mail: adam.stenman@ki.se

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Introduction

Telomerase reverse transcriptase (TERT) is a subunit of the telomerase enzyme complex, which can increase telomere length by adding TTAGGG repeats (1,2). As telomeres become shorter for each cell division, this addition of nucleotides increases the possible number of cell divisions. In cancer, telomerase activation is frequently observed. *TERT* promoter mutations have been associated with more aggressive disease and therefore suggested as a prognostic marker (2). Shorter telomeric repeat sequences are commonly observed in tumor tissues, further supporting the telomere involvement (3).

Adrenocortical carcinoma (ACC) is a rare endocrine tumor with a generally poor prognosis. The majority of patients present with metastatic or inoperable disease, and despite initial tumor-free resection margin surgery, a majority of patients relapse or develop progressive disease (4). Mitotane (Lysodren) is used as a primary therapy and adjuvant treatment (5); however, it has serious and ill-tolerated side effects (6). The majority of cases of ACC arise in a sporadic setting and exhibit frequent somatic mutations in multiple genes, including the driver genes tumor protein 53 (*TP53*), catenin β1, zinc and ring dinger 3, and protein kinase cAMP-dependent type I regulatory subunit α (7-9).

The upregulation of *TERT* and telomerase activity have previously been reported in ACC (10,11). One genetic mode for the upregulation of *TERT* stems from the recurrent *TERT* promoter mutations C228T and C250T, which were first described in patients with melanoma (12). Such mutations have been found in ACC, providing a possible explanation for the observed expression of *TERT* in subsets of this tumor type. However, as the expression of *TERT* was also found in *TERT* wild-type cases, additional genetic or epigenetic mechanisms

are expected to be involved (10). In ACC, *TERT* copy number gain has been found as a recurrent genetic event and mutually exclusive from established driver gene mutations (7). However, the occurrence and impact on disease presentation of epigenetic modifications, including *TERT* promoter methylation, remains to be elucidated in ACC. Studies of other cancer types have identified regions of high methylation in association with poor patient outcome, including at positions -578 to -541 bp in medullary thyroid carcinoma (Region A, Fig. 1A) (13), and a region located -162 to -100 bp infront of the transcriptional start site (Region B, Fig. 1A) in which low methylation was shown to be required for the expression of *TERT in vitro* (14).

In the present study, *TERT* promoter methylation density, copy number and telomere length were assessed in relation to clinical characteristics in ACC samples, which were also characterized for *TERT* promoter mutations and *TERT* gene expression.

Patients and methods

Tumor samples and references. Samples were collected in accordance with the ethical permits obtained from the Ethical Committee of Karolinska Institutet (Stockholm, Sweden) and from the International Review Board at Yale University (New Haven, CT, USA). Tissue material was collected with informed consent obtained from patients prior to surgery.

In total, 38 tumor samples were used in the experiments. Of the tumors, 27 (ACC-1-27) were collected and diagnosed at the Karolinska University Hospital (Stockholm, Sweden) between July 1986 and July 2009. In this cohort, 16 were female and 11 were male, with an age range of 28 to 84 years old (mean age, 59.4 years). Six patients had metastases at the time of surgery. Samples were snap frozen in liquid nitrogen in conjunction with postoperative pathological grossing. All of these tissues were histopathologically confirmed and samples with <50% tumor cells were excluded from the study. The remaining 11 samples (ACC-Y1-Y11) were collected between August 2003 and July 2015 at Yale University. In this cohort, 8 patients were female and 3 were male, with an age range of 18 to 69 years (mean age, 52.2 years) at surgery. Two of the patients had metastasis at the time of surgery. Genomic DNA was extracted from formalin-fixated paraffin-embedded samples.

The following adrenal references were used for the investigation of methylation levels and the devaluation of relative telomere length: Eight histologically confirmed normal adrenal tissue samples (comprising a majority of adrenocortical cells), from renal or adrenal surgical specimens; two commercially available adrenal medullary DNA (Clinomics, Watervliet, NY, USA); (NAT, n=10); and adrenocortical hyperplasia (ACH) tissue samples (n=8, only for relative telomere length). For the copy number analysis, 14 histologically confirmed NAT samples were used in addition to the two commercially available adrenal medullary DNA (NAT=16).

The tumors were diagnosed according to the criteria of the 2004 World Health Organization classification (15). The ACC cases were clinically characterized for sex, age at surgery, tumor size (largest diameter), tumor weight, MIB1 proliferation index (based on Ki-67 immunohistochemistry) European Network for the Study of Adrenal Tumors (ENSAT) staging score, metastasis, and outcome. Some of the clinical information has been previously published (7,10).

TERT promoter mutation status. Of the tumors, 27 were previously screened for the *TERT* promoter hot spot mutations C228T and C250T and investigated for quantification of the mRNA expression of *TERT* (ACC-1-27; Table I) (10). The other 11 samples (ACC-Y1-Y11) were investigated for *TERT* promoter mutation status and the analysis was successfully performed for eight of these samples. The mutation status was determined using Sanger sequencing, applying a previously described methodology (10), which revealed that one of the eight samples showed the C228T mutation and the remaining seven samples were wild-type (Table I).

DNA copy number. Genomic DNA was extracted from tumour tissues using a DNAeasy Blood and Tissue DNA isolation kit (Qiagen AB, Sollentuna, Sweden). A total of 27 ACC samples was successfully analyzed for *TERT* copy numbers. DNA at 5 ng/μl was analyzed (with five repeats) with the TaqMan assays (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) Hs 01237576_CN for *TERT*, with Hs 4403326_C for the *RNaseP* gene used as an endogenous control. Following normalization to *RNaseP*, data were calibrated to NAT DNA. The data were exported to CopyCaller v2.0 (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the copy numbers were estimated.

Pyrosequencing. The *TERT* promoter regions investigated for methylation density are shown in Fig. 1A. CpG methylation was quantified at eight sites within Region A located between -578 and -541 bp, and five sites within Region B between -162 and -100 bp. DNA from a total of 35 ACC samples was successfully analyzed for Region A, and from 37 ACC samples for Region B.

The methodology for pyrosequencing was as previously described (13), and the primers used are detailed in Table II. For Region B, the primers were designed in-house with PyroMark Assay Design 2.0 (Qiagen AB). The majority of samples were fresh frozen following surgery and a minority of the samples was paraffin-embedded. Previous studies have demonstrated the validity of both materials in sequencing experiments (16). Bisulphite treatment was performed with the EpiTect bisulphite kit (Qiagen AB). Polymerase chain reaction (PCR) amplification of the bisulphite converted DNA was performed with the Qiagen PCR kit (Qiagen AB), including 20 ng DNA, 12.5 μl PyroMark PCR Master mix, 2.5 μl Coral load, 7 μl H_2O, 1 μl $MgCl_2$ and 0.5 μl primers 10 μM for Region A (forward, 5'-GGGTTTGTGTTAAGGAGTTTA AGT-3' and reverse, 5'-AAACCCAAAACTACCTCCA-3') and 5 μM for Region B (forward, 5'-GGTGGTAGGGGTTAG GGTTTTTTA-3' and reverse, 5'-TACCCCTTCACCTTCCAA CTC-3'). PCR amplifications included 45 cycles of 30 sec at 94˚C, 30 sec at 58˚C for Region A and at 56˚C for Region B, and 30 sec at 72˚C. The pyrosequencing reactions were run in a PyroMark Q24 system and the data were analyzed with PyroMark Q24 2.0.7 software (Qiagen AB). For each sample, a methylation index (MetI) was calculated as the mean methylation density of the investigated CpGs for Region A and Region B, respectively. For survival analysis, the cut-off for

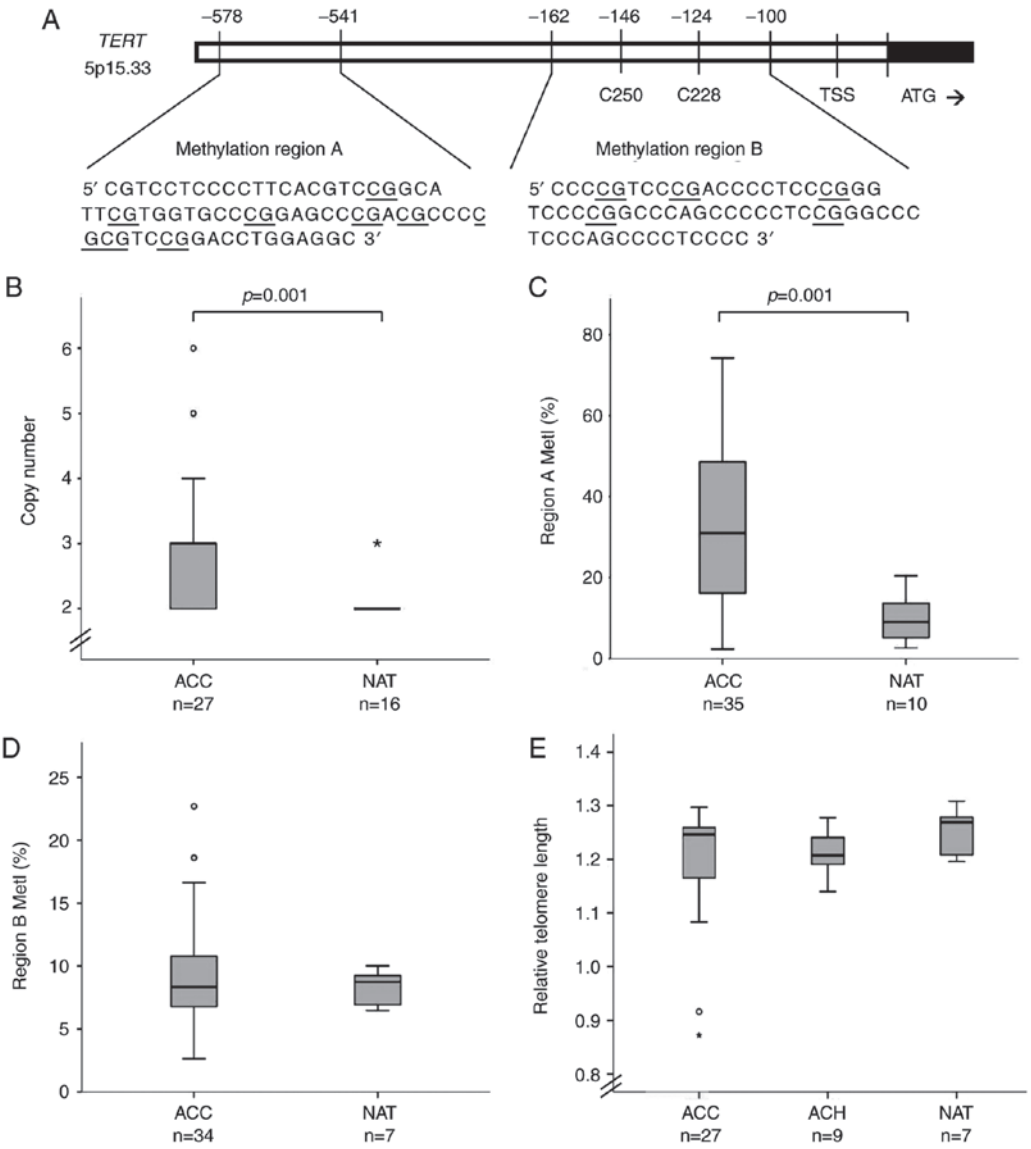

Figure 1. *TERT* promoter methylation sites and boxplot presentation of *TERT* promoter methylation densities, *TERT* copy number analysis and telomere length investigation. (A) Schematic illustration of the *TERT* promoter region in chromosomal region 5p15.33. Investigated CpG sites are underlined. The common mutation sites C250 and C228 are indicated. ATG represents the start of translation, and TSS the transcription start site. (B) *TERT* copy numbers for ACC tissues and NAT samples. (C) MetI of Region A for ACC and NAT samples. (D) MetI of Region B for ACC and NAT samples. (E) Relative telomere lengths in ACC, ACH and NAT samples. Boxes correspond to interquartiles, bars to non-outlier ranges, and bold lines to medians. Outliers are indicated by circles and extreme values by stars. A broken vertical axis is used for graphs B and E. *TERT*, telomerase reverse transcriptase; ACC, adrenocortical carcinoma; NAT, normal adrenal tissue; ACH, adrenocortical hyperplasia; MetI, methylation index.

high and low methylation in Region A was set at 20.4% based on the highest methylation level for NAT.

Telomere length. The relative telomere length was determined for the 27 ACC samples using a previously described methodology (17,18) and the primers are detailed in Table II. DNA at 10 ng/μl was used to determine the mean relative telomere length of the tumors using quantitative (q)PCR amplification of telomere (*TEL*) and β-globin (*HBG*), the latter serving as an internal control. The following was included in the experiment: 5 μl SYBR® Green Real-Time PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific Inc.), 0.2 μl primers (*TEL* forward, 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3' and reverse, 5'-GGCTTGCCTTACCCTTACCCTTACCC-TTACCCTTACCCT-3'; *HBG* forward, 5'-TGTGCTGGCCCATCACTTTG-3' and reverse, 5'-ACCAGCCA-CCACTTTCTGATAGG-3') in 10 μM, 2.6 μl H_2O and 20 ng DNA. The thermocycling conditions were as follows: 50˚C for 2 min, 95˚C for 2 min, 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec. All samples were run in triplicate. The data was analyzed in Excel 2010 (Microsoft Corporation, Redmond, WA, USA), whereby the mean Cq value of each sample was used in the calculations. Outliers were manually omitted. The ratio of *TEL* compared to *HBG* was used to estimate the relative telomere length and was used for comparison between samples.

Statistical analyses, graphs and figures. Statistical analyses was performed and figures made using IBM SPSS 23 (IBM SPSS, Armonk, NY, USA). The results of MetI at

Table I. *TERT* genetic and epigenetic data and telomere length in 38 cases of ACC.

Case	Cohort	*TERT* copy no.	*TERT* mutation[a]	*TERT* promoter MetI % (range)		*TERT* mRNA[a]	Relative telomere length
				Region A	Region B		
ACC-1	Karolinska	2	No	25 (5-40)	7 (5-10)	0.000389	0.92
ACC-2	Karolinska	2	No	31 (7-49)	19 (7-52)	0	0.87
ACC-3	Karolinska	3	No	50 (40-60)	8 (5-13)	0.049385	1.26
ACC-4	Karolinska	2	No	20 (10-27)	6 (4-9)	0.007639	1.21
ACC-5	Karolinska	3	No	8 (3-15)	-	0.017604	1.27
ACC-6	Karolinska	2	No	3 (1-4)	6 (3-10)	0.000408	1.17
ACC-7	Karolinska	2	C228T	41 (9-63)	-	0.001654	1.20
ACC-8	Karolinska	3	No	37 (30-41)	7 (3-11)	1.349172	1.25
ACC-9	Karolinska	5	No	46 (24-71)	10 (5-17)	0.000217	1.15
ACC-10	Karolinska	6	No	34 (8-71)	8 (5-11)	0.029137	1.23
ACC-11	Karolinska	3	No	8 (3-14)	8 (3-15)	0.269476	1.16
ACC-12	Karolinska	2	C228T	12 (6-21)	8 (5-13)	0.016867	1.26
ACC-13	Karolinska	2	No	45 (15-60)	11 (7-18)	0.000207	1.23
ACC-14	Karolinska	4	No	46 (19-70)	11 (5-24)	0.000997	1.26
ACC-15	Karolinska	3	No	57 (9-90)	9 (5-14)	0.000196	1.08
ACC-16	Karolinska	4	No	49 (29-62)	6 (4-10)	0.00856	1.25
ACC-17	Karolinska	3	No	31 (12-51)	6 (3-10)	0.00027	1.25
ACC-18	Karolinska	3	No	14 (6-22)	8 (5-14)	0.44975	1.30
ACC-19	Karolinska	2	No	48 (12-88)	6 (3-8)	0.292925	1.25
ACC-20	Karolinska	2	No	31 (9-68)	17 (5-30)	0	1.11
ACC-21	Karolinska	3	No	74 (61-87)	8 (5-10)	0.010663	1.28
ACC-22	Karolinska	3	No	60 (40-85)	10 (4-14)	0.019338	1.17
ACC-23	Karolinska	3	No	19 (8-39)	9 (6-14)	0.001294	1.25
ACC-24	Karolinska	2	No	13 (2-25)	9 (7-11)	0.000382	1.25
ACC-25	Karolinska	3	No	10 (2-17)	14 (7-20)	0	1.15
ACC-26	Karolinska	3	No	30 (6-60)	11 (6-14)	0.007517	1.27
ACC-27	Karolinska	2	C228T	28 (10-54)	-	0.006031	1.29
ACC-Y1	Yale	-	No	52 (45-68)	7 (3-10)	-	-
ACC-Y2	Yale	-	No	74 (66-83)	9 (1-24)	-	-
ACC-Y3	Yale	-	No	13 (2-33)	13 (4-33)	-	-
ACC-Y4	Yale	-	No	19 (3-44)	12 (1-22)	-	-
ACC-Y5	Yale	-	No	51 (40-96)	7 (1-19)	-	-
ACC-Y6	Yale	-	C228T	19 (14-30)	5 (2-8)	-	-
ACC-Y7	Yale	-	No	73 (40-89)	23 (8-73)	-	-
ACC-Y8	Yale	-	-	2 (1-4)	-	-	-
ACC-Y9	Yale	-	-	-	5 (1-19)	-	-
ACC-Y10	Yale	-	-	-	10 (1-43)	-	-
ACC-Y11	Yale	-	No	-	3 (2-4)	-	-

[a]Data for ACC 1-27 from Ref. (10). -, not determined or not informative; MetI, methylation index; *TERT*, telomerase reverse transcriptase; ACC, adrenocortical carcinoma.

Region A and B, *TERT* copy number and telomere length in the sample groups were compared with clinical parameters, *TERT* mutation status and expression. In addition, statistical analyses of *TERT* promoter methylation and clinical parameters, and survival rates were performed. The Mann-Whitney U test and Kruskal-Wallis test were used for comparison between groups. Correlations were assessed using Spearman's rank order. Disease-related and overall survival rates were analyzed using the Log-rank test and illustrated with Kaplan-Meier plots. $P<0.05$ was considered to indicate a statistically significant difference.

Table II. Primers used for methylation and telomere length analyses.

Gene primer	Primer direction	Primer sequence
Promoter methylation region A		
TERT	Forward	5'-GGGTTTGTGTTAAGGAGTTTAAGT-3'
TERT	Reverse	5'-AAACCCAAAACTACCTCCA-3'
TERT	Sequencing	5'-CCAAAACTACCTCCAAAT-3'
Promoter methylation region B		
TERT	Forward	5'-GGTGGTAGGGGTTAGGGTTTTTTA-3'
TERT	Reverse	5-TACCCCTTCACCTTCCAACTC-3'
TERT	Sequencing	5'-GGGGTAGAGGAAAGGAA-3'
Relative telomere length		
Tel 1b	Forward	5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3'
Tel 2b	Reverse	5'-GGCTTGCCTTACCCTTACCCTTACCC-TTACCCTTACCCT-3'
HBG1	Forward	5'-TGTGCTGGCCCATCACTTTG-3'
HBG2	Reverse	5'-ACCAGCCA-CCACTTTCTGATAGG-3'

Primers for telomere length are according to Ref (18). *TERT*, telomerase reverse transcriptase; *Tel*, telomere.

Results

Evaluation of the TERT gene locus. The *TERT* gene locus was investigated for methylation densities at Region A and Region B, in addition to DNA copy numbers and telomere length in a panel of ACC samples. The overall results from these investigations are shown in Fig. 1B-E and Table I. The acquired results were subsequently used in statistical comparisons with clinical data, in addition to data for *TERT* promoter mutations and the gene expression of *TERT* (Table III).

TERT copy number gain in ACC. The mean *TERT* copy number of 2.9 was observed for the ACCs (range 2-6, median 3) and of 2.1 for the NATs (Fig. 1B). The ACCs had a significantly higher copy number, compared with the NATs (P=0.001, Mann-Whitney U test) (Figs. 1B and 2). In total, 19/27 cases (70%) exhibited either copy number gain (16/27, 59%) or promoter mutation (3/27, 11%) (Fig. 2), without overlap. In 24/27 ACCs (89%) *TERT* mRNA expression was present, however, no association was observed between the expression of *TERT* and copy number gain or promoter mutation.

Association of high methylation in Region A with poor outcome and advanced disease. The mean MetI in Region A was 33.5% in the ACCs (range 2.3-74.2%, median 31.0%) and 10.2% in the NATs (range 2.6-20.4%, median 9.0%). Similar patterns were observed with regard to the individual CpGs investigated (Fig. 2B).

The ACC group had a significantly higher MetI, compared with the NAT group (P=0.001, Mann-Whitney U test) (Figs. 1C and 2). No correlation was observed between Region A MetI and the mRNA expression levels of *TERT*.

Comparisons with clinical characteristics of the ACC samples revealed that higher MetIs for Region A were associated with metastases or relapse of disease (P<0.01, Mann-Whitney U test), with poor patient outcome (P<0.05, Mann-Whitney U test) and higher ENSAT stage (r=0.39; P<0.05, Spearman's rank order correlation) (Table III; Fig. 3A and B). Patients with MetI >20.4% had shorter disease-related survival and absolute survival rates (P<0.05, Log-rank test), compared with the patients with MetI <20.4% (Fig. 3C and D).

Association between methylation levels in Region B and the mRNA expression of TERT in ACC. In Region B, a mean MetI of 9.3% was observed in the ACC group (range 2.6-22.7%, median 8.3%) and of 8.2% in the NAT group (range 6.5-10.0%, median 8.8%). Overall, no difference in MetI was observed between the ACC and NAT groups (Fig. 1D; Table I).

In the ACCs, MetI of Region B was inversely correlated with the mRNA expression levels of *TERT* (P<0.05, r= -0.554, Spearman's rank order correlation, Table III). All of the 24 ACC samples that exhibited mRNA expression of *TERT* had a MetI <11.25%, and the highest MetIs were noted in the three ACC samples without traceable expression of *TERT* (Fig. 2A and B). The difference was particularly pronounced for CpG number 2 (Fig. 2B).

Correlation between relative telomere length and mRNA expression of TERT in ACC. The relative telomere lengths were determined, which revealed a mean of 1.20 in the ACC group (range 0.9-1.3, median 1.25), of 1.21 in the ACH group (range 1.14-1.28, median 1.2) and of 1.25 (range 1.20-1.31, median 1.27) in the NAT group. No difference was observed between the ACC group and the NAT or ACH group (Fig. 1E). In the ACC group, the relative telomere length was positively correlated with the mRNA expression of *TERT* (r=0.57, P<0.05, Spearman's rank correlation, Table III). Furthermore, shorter telomere length was associated with poorer outcome (P<0.05, Mann Whitney U test; Table III).

Discussion

Telomerase activity has been shown to be present in ACC (19). *TERT* promoter mutations, which may be one underlying

Table III. Summary of statistical analyses.

Statistical test parameter	Copy number	Region A MetI	Region B MetI	Telomere length
Mann-Whitney U test				
TERT mutation	**0.044**	0.285	0.213	0.247
Outcome	0.521	**0.033**	0.382	**0.027**
Absolute outcome	0.555	0.051	0.086	**0.048**
Metastasis/relapse	0.732	**0.008**	0.358	0.133
Sex	0.668	0.657	0.330	0.921
Spearman's rank order correlation				
Tumor weight	0.152	0.623	0.349	0.573
Tumor size	0.196	0.756	0.153	0.936
ENSAT stage	0.714	**0.021**	0.142	0.566
Ki-67 index	0.949	0.107	0.476	0.807
TERT mRNA	0.224	0.79	**0.005**	**0.002**

Statistically significant P-values (P<0.05) are indicated in bold. MetI, methylation index; ENSAT, European Network for the Study of Adrenal Tumors.

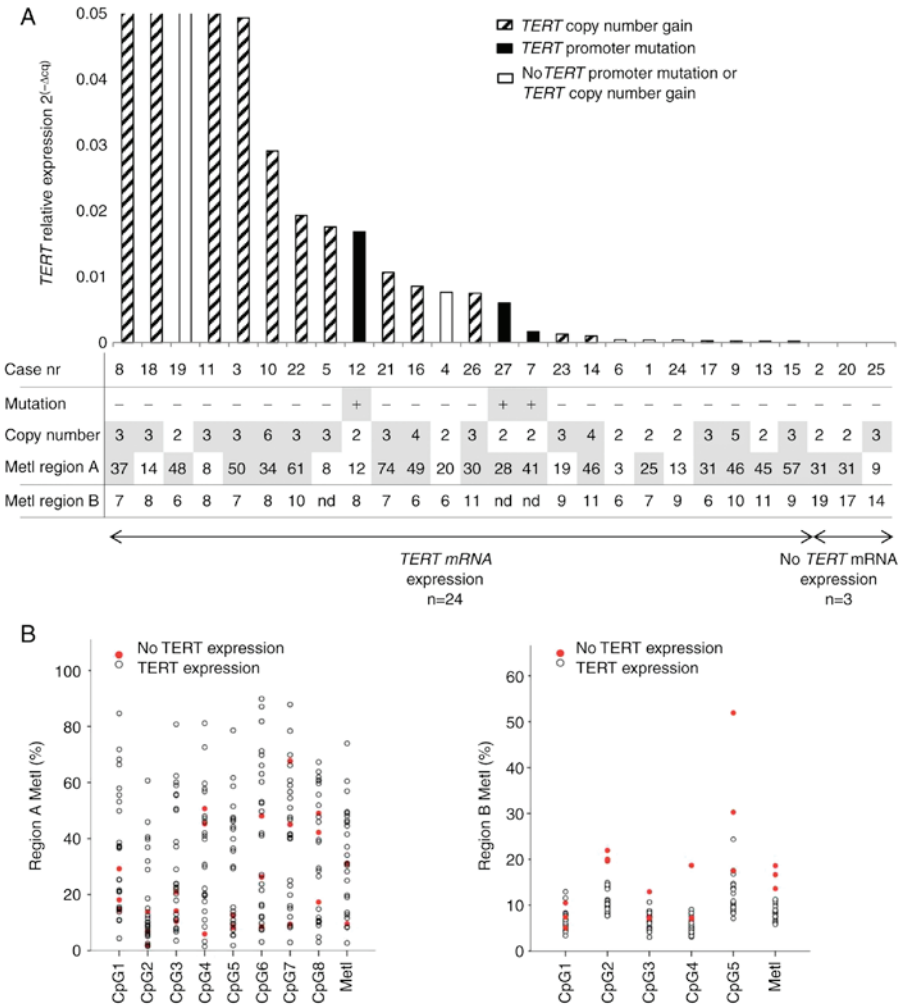

Figure 2. Results of expression of *TERT*, copy number gain, mutation status and promoter methylation in Regions A and B in 27 ACC cases. (A) Relative mRNA expression of *TERT* is shown at the top. Cases with a *TERT* promoter mutation are indicated by solid bars, cases with *TERT* copy number gain by striped bars, and cases without *TERT* promoter mutation or *TERT* copy number gain by unfilled bars. *TERT* mutations, copy numbers, and MetI (%) for Regions A and B in the same ACC cases are shown below. Cases with mutation, copy number gain and increased MetI in Region A are highlighted (cut-off at >20.4%). (B) Scatterplots for 27 ACC samples with known *TERT* expression. Methylation densities are shown at specific CpG sites in addition to overall MetI in Region A and B. Tumors with and without *TERT* mRNA expression are indicated as red and open circles, respectively. *TERT*, telomerase reverse transcriptase; ACC, adrenocortical carcinoma; MetI, methylation index; nd, not determined.

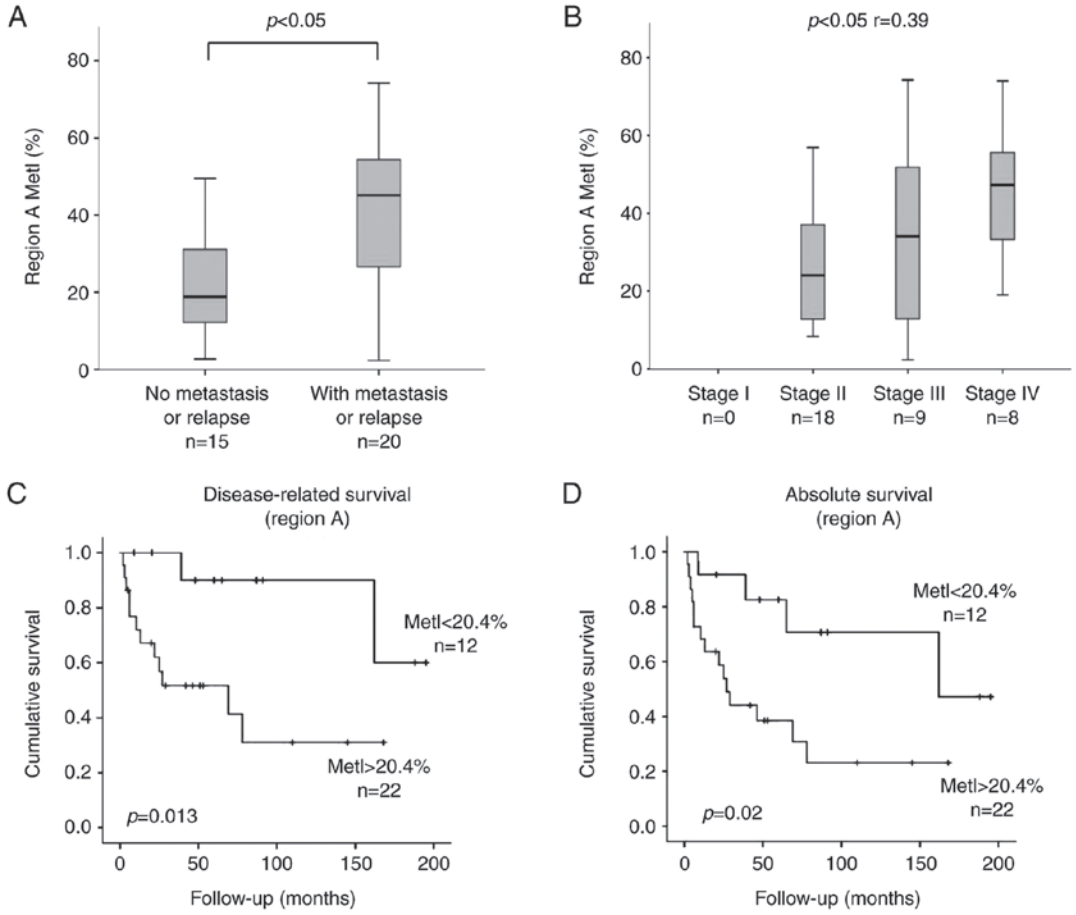

Figure 3. Boxplot presentation of *TERT* promoter methylation for Region A in relation to metastasis/recurrence and stage in ACC and Kaplan-Meier plots for survival analysis of ACC cases according to Region A *TERT* promoter methylation. (A) MetI in Region A according to the presence or lack of metastases or relapse in 35 ACC cases. (B) MetI in Region A according to Stage I, II, III and IV disease based on European Network for the Study of Adrenal Tumor score in 35 ACC cases. (C) Disease-related survival for 34 ACC cases. (D) Absolute survival for 34 ACC cases. Increased and decreased MetI in Region A refers to above and below the cut-off at 20.4%, respectively. *TERT*, telomerase reverse transcriptase; ACC, adrenocortical carcinoma; MetI, methylation index; nd, not determined.

mechanism behind this telomerase activity, are known to occur in up to 10% of ACC cases and *TERT* expression is detected in the majority of tumors. Therefore, additional mechanisms are expected to be involved in the activation. Copy number gain and aberrant promoter methylation are additional frequently occurring alterations in this tumor entity. Genetic and epigenetic *TERT* alterations were present in the majority of ACC samples analyzed in the present study, supporting the hypothesis that the development of *TERT*-based diagnostic tools may be of value for improved diagnostics of this entity. Furthermore, increased methylation density in Region A was associated with metastatic disease, higher ENSAT stage and shorter survival rates, which may be further developed into an additional prognostic tool for ACC. Given the toxic effects of mitotane on the gastrointestinal system (anorexia, diarrhea, nausea, vomiting), the central nervous system (apathy, drowsiness, neuropathy, dizziness) and to the hormonal system (adrenal insufficiency) (6), a novel prognostic tool for ACC may be of important clinical significance for therapeutic decisions.

Gain of the *TERT* locus in chromosomal region 5p13.3 is a common event in ACC, which can be observed at the chromosome level (20) and for the individual gene locus (7-9). In the present study, increased *TERT* copy numbers were detected in 16/27 (59%) of the ACC samples. These predominantly included gains, whereas amplifications were observed in a small number of tumors. These findings are in agreement with previous studies in which increased copy numbers were reported either in the form of gains, amplifications or both (7-9).

In the present study, *TERT* copy number gain or promoter mutations were present in 70% of ACC samples in a non-overlapping manner. The *TERT* mutation has previously been associated with induction of the expression of *TERT* (12). Copy number aberrations are characteristic of ACC but uncommon in adrenocortical adenomas (20). A copy number increase of the telomerase encoding regions has been suggested as a possible explanation for telomerase activation (21). This type of copy number gain has been found in several tumor types, and the components that build up the telomerase complex are located on chromosomal regions, which have been reported with frequent copy number increase (21). As *TERT* promoter mutations are known to confer increased transcription of *TERT*, it is likely that *TERT* copy number gain or mutation may each contribute to the observed expression.

Epigenetic modifications, including DNA methylation of CpG sites, are major contributors to the construction and

organization of the DNA molecule and also have an impact on gene expression. Altered CpG methylation has been shown in several cancer types and is a proposed therapeutic target (22), however, methylation densities in the *TERT* promoter of ACC have not been investigated despite lacking knowledge of the underlying cause for increased expression of *TERT* in these tumors. In the present study, high MetI in Region A was found to be associated with metastases and poorer survival rates in patients with ACC. Further evidence of this connection was shown by the association between MetI for Region A and ENSAT stage, where a higher ENSAT stage indicates more advanced cancer development. These findings are in line with previous observations in medullary thyroid carcinoma, where high MetI was associated with a shorter survival rate (13). Future investigations are required to investigate MetI in association with telomerase activity in ACC, which may provide additional information on the role of methylation levels in the *TERT* promoter.

A previous study (23) examined the methylation levels of CpG islands in ACC and found that genome-wide hypermethylation was associated with poor survival rate. However, the *TERT* locus was not investigated. The findings reported by Jouinot *et al* (23) suggest that *TERT* promoter hypermethylation at Region A may be due to a general hypermethylation phenotype. If so, *TERT* hypermethylation in Region A may be one of the important effects of such a profile due to its ability to influence telomere length and promote tumor cell survival. These results indicated the prognostic value of investigating methylation in this tumor type, which was further recognized in the results of the present study.

In the cohort of ACC cases used in the present study, the gene expression of *TERT* was found in 90% of the ACC cases. The majority of these tumors harbored *TERT* copy number gain, the C228T promoter mutation, or increased MetI in Region A, suggesting that these alterations contributed to the gene expression of *TERT*. No significant associations were revealed between the expression levels of *TERT* and copy numbers, mutation or MetI for Region A. This suggested that several different mechanisms are involved in the acquired increase of transcription, and that *TERT* copy number gain and increased *TERT* promoter methylation are two elements of the upregulation of *TERT*. Additional investigations are required to further understanding of the underlying mechanisms of the upregulation of *TERT*.

An inverse correlation was revealed between MetI for Region B and the expression of *TERT*. Low methylation in this region has been shown to be important for the transcription of *TERT* to occur *in vitro* by affecting the binding of the 11-zinc finger protein transcription factor CTCF (14). All ACC samples exhibiting *TERT* expression had low MetI at Region B. By contrast, the highest levels of Region B MetI were revealed in the three ACC samples in which the *TERT* gene was not expressed. Taken together, these observations supported the hypothesis that low methylation close to the transcription start site is a requisite for the transcription of *TERT* in the *in vivo* ACC tumor situation in addition to previous observations *in vitro*.

Telomere length and cancer development are considered to be associated, and telomere length in tumors and in constitutional tissues of cancer patients have been associated with cancer development and survival rates. For ACC, this has been supported by observations in Li-Fraumeni syndrome, in which the development of malignancies, including ACC, has been associated with shorter telomeres in *TP53* mutation carriers (24). Induction of the expression of *TERT* is a major contributor to telomerase activation and subsequent elongation of telomeres. In line with this, the expression of *TERT* in ACC was positively correlated with telomere length, and telomere length was associated with patient survival rates. In the present study, mean relative telomere length was calculated using a qPCR-based method, which makes the results suitable for comparison only between tumors of this study. A sequencing-based method for calculating telomere length was used (8) for ACC, which found that the majority of ACC samples had shorter telomeres compared with matching normal samples. In the present study, a similar effect on telomere length was not observed, however the comparison was performed against independent NAT samples. In the same previous study, the results showed no association between telomere length and *TERT* mutation or *TERT* amplification, which is in accordance with the results of the present study (8).

Activation of a telomere-maintaining mechanism is a feature of ACC (11,25), but has not been consistently found in adrenocortical adenomas or NAT (26). One mechanism that is expected to be operative in *TERT*-positive ACCs, is the activation of telomerase when the telomere length hits a critical low point. In the present study, 90% of ACC samples were positive for the expression of *TERT*, which is in agreement with a report of frequent telomerase activation in ACC based on a TRAP assay (11). Besides telomerase activation, homologous recombination has been described in elongation of telomeres in cancer, termed alternative lengthening of telomeres (ALT). Although telomerase activation is the common mechanism of telomere maintenance in ACC, a small number of ACC cases have been shown to utilize the ALT mechanisms or, rarely, both (11).

Although the present study did not demonstrate a significant association between *TERT* promoter methylation density, *TERT* copy number gain and expression of *TERT*, the increase in MetI and copy number was widely observed within these tumors, compared with NAT samples, and MetI in Region A was associated with poorer clinical outcome, which indicated that these alterations are of importance in this type of tumor. In conclusion, the present study provided evidence for epigenetic alterations of the *TERT* gene locus being of significant importance for the severity of ACC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FS, AS, CCJ and CL conceived and designed the study. FS, JOP, OF, NW and NM researched and analysed data. TDM, RK, TC, MB and CCJ provided tissue samples and clinical details. FS, AS, CCJ and CL wrote, edited and reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All tissue samples were collected with informed consent and ethical permission granted from the Ethics Committee of Karolinska Institutet and from the International Review Board at Yale University.

Patient consent for publication

Not applicable.

Competing interests

The authors confirm that they have no competing interests.

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