Cancer Cell International

RESEARCH

Open Access

TERT upstream promoter methylation regulates TERT expression and acts as a therapeutic target in *TERT* promoter mutation-negative thyroid cancer

Shiyong Li[1], Guanghui Hu[1], Yulu Chen[1], Ye Sang[1], Qin Tang[1] and Rengyun Liu[1*]

Abstract

Background DNA hypermethylation and hotspot mutations were frequently observed in the upstream and core promoter of *telomerase reverse transcriptase* (*TERT*), respectively, and they were associated with increased TERT expression and adverse clinical outcomes in thyroid cancer. In *TERT* promoter mutant cancer cells, the hypomethylated *TERT* mutant allele was active and the hypermethylated *TERT* wild-type allele was silenced. However, whether and how the upstream promoter methylation regulates TERT expression in *TERT* mutation-negative cells were largely unknown.

Methods DNA demethylating agents 5-azacytidine and decitabine and a genomic locus-specific demethylation system based on dCas9-TET1 were used to assess the effects of *TERT* upstream promoter methylation on TERT expression, cell growth and apoptosis of thyroid cancer cells. Regulatory proteins binding to *TERT* promoter were identified by CRISPR affinity purification in situ of regulatory elements (CAPTURE) combined with mass spectrometry. The enrichments of selected regulatory proteins and histone modifications were evaluated by chromatin immunoprecipitation.

Results The level of DNA methylation at *TERT* upstream promoter and expression of TERT were significantly decreased after treatment with 5-azacytidine or decitabine in *TERT* promoter wild-type thyroid cancer cells. Genomic locus-specific demethylation of *TERT* upstream promoter induced TERT downregulation, along with cell apoptosis and growth inhibition. Consistently, demethylating agents sharply inhibited the growth of thyroid cancer cells harboring hypermethylated *TERT* but had little effect on cells with *TERT* hypomethylation. Moreover, we identified that the chromatin remodeling protein CHD4 binds to methylated *TERT* upstream promoter and promotes its transcription by suppressing the enrichment of H3K9me3 and H3K27me3 at *TERT* promoter.

Conclusions This study uncovered the mechanism of promoter methylation mediated TERT activation in *TERT* promoter mutation-negative thyroid cancer cells and indicated *TERT* upstream promoter methylation as a therapeutic target for thyroid cancer.

Keywords DNA methylation, Telomerase reverse transcriptase, Gene regulation, Chromatin, Thyroid cancer

*Correspondence:
Rengyun Liu
liury9@mail.sysu.edu.cn

Full list of author information is available at the end of the article

Background

Thyroid cancer increased rapidly over the past few decades, and now it has become the most common endocrine malignancy and ranks in the 7th place among all types of cancer [1]. A number of genetic variants and epigenetic alterations orchestrate the initiation and progression of thyroid cancer by upregulating the expression of some oncogenes and downregulating the expression of tumor suppressor genes. It has been well recognized that most of thyroid cancer cases carry genetic alterations in the MAPK and PI3K/AKT pathways, and somatic mutations and gene fusions were frequently occurred in several driver genes like *BRAF*, *RAS*, *RET*, *PIK3CA* and *EIF1AX* [2–4]. As the most widely investigated epigenetic modification, DNA methylation had been well proved to contributes to thyroid cancer by regulating the expression of thyroid-specific genes, transcriptional factors, and several tumor suppressor genes [5, 6].

Telomerase reverse transcriptase (TERT), the catalytic subunit of telomerase, function as an oncogene in human cancers through telomere -dependent and -independent manners [7]. It is undetectable in normal tissues, but constitutively reactivated in 85–90% of cancer cases. Earlier studies revealed that several transcriptional factors, including c-MYC and SP1, were capable of activating TERT transcription by directly binding to its promoter in cancer cells [8]. Recently, two hot-spot somatic mutations in the core promoter region of *TERT* were identified in several types of cancer, including thyroid cancer [9]. And they constructively activated TERT expression by recruiting ETS transcriptional factors [10–13]. In addition, the expression of TERT was regulated by histone modifications, non-coding RNA and DNA methylation [14].

Interestingly, DNA methylation in the promoter region is well known to silence gene's expression, while the *TERT* promoter region was high methylated in tumor samples in multiple types of cancer, particular in a 433-bp region encompassing 52 CpG sites located upstream the core promoter, which was termed as *TERT* hypermethylated oncological region (THOR) [15]. Increasing studies have established an association between *TERT* promoter hypermethylation and high expression of TERT, aggressive characteristics and/or poor prognosis in several types of cancer, including glioma, prostate cancer, pancreatic cancer, bladder cancer, breast cancer, and thyroid cancer [16–26]. An allele-specific model of TERT regulation was recently established in *TERT* promoter mutant cancer cells based on findings that the activated *TERT* mutant allele was hypomethylated while the silenced wild-type allele was frequently hypermethylated [27–31]. However, the regulatory effect of *TERT* upstream promoter methylation on the gene's expression remains elusive in cancer cells that do not harbor *TERT* promoter mutation. Herein, this study aimed to explore whether and how DNA methylation at the upstream region of *TERT* promoter regulates its expression in *TERT* promoter mutation-negative thyroid cancer cells.

Methods

Cell lines and demethylating agents

Thyroid cancer cell lines TTA1, CAL62, WRO and human embryonic kidney 293T cells were purchased from the National Collection of Authenticated Cell Cultures (Shanghai, China), the thyroid cancer cell line MDA-T41 was purchased from American Type Culture Collection (Manassas, VA, USA). All the cells were cultured in DMEM medium (#10-013-CVRC, Corning) with 10% fetal bovine serum (#04-001-1A, Biological Industries) under the condition of 5% CO_2 and 37 °C. The mutational status at *TERT* core promoter region were examined as we described previously [32], and all these cell lines were negative for *TERT* promoter mutation. The DNA demethylating drugs 5-Azacytidine (#A2385) and decitabine (5-Aza-2'-deoxycytidine, #A3656) were purchased from Sigma-Aldrich. These drugs were dissolved in DMSO and used for treating cells at different concentrations for 1–5 days as indicated based on specific research purpose.

Bisulfite sequencing

Genomic DNA was extracted from cultured cells using the EZNA Tissue DNA Kit (#D3396-02, Omega Bio-tek) and subjected to sodium bisulfite modification using the EZ DNA Methylation Lightning Kit (#D5031, Zymo Research) according to the manufacturer's instructions. The *TERT* promoter region was amplified by PCR using ZymoTaq PreMix (#E2004, Zymo Research) with two pairs of primers (Additional file 1). The primer pair F1/R1 was used for amplifying the −175 to -466 bp of *TERT* promoter, the primer pair F2/R2 was used for amplifying the −430 to -840 region of *TERT* promoter. The PCR products were then cloned to T vector and subjected to Sanger sequencing.

RNA extraction and quantitative PCR (qPCR) analysis

Total RNA was extracted from cultured cells using the TRIzol reagent (#15596018, Invitrogen) and then reverse-transcribed to cDNA using the RevertAid First Strand cDNA Synthesis Kit (#K1622, ThermoFisher). Gene expression was detected in triplicate using the PowerUp SYBR Green Master Mix (#A25742, Applied Biosystem). β-Actin was used as the internal control for normalization. The primers used for qPCR amplification are listed in Additional file 1. Relative mRNA expression of the aimed genes was calculated according to the $2^{-\Delta\Delta}$CT method.

Gene knockdown

The short hairpin RNA (shRNA) specifically against *PRKDC*, *SMC1a*, *CND2*, *TF3C2* and *CHD4* as well as the scramble control shRNA (Additional file 2) were cloned into the lentiviral vector pLKO.1-puro (#8453, Addgene). The lentiviral shRNA-expressing vector together with the packaging plasmid psPAX2 (#12260, Addgene) and the VSV-G envelope expressing plasmid pMD2.G (#12259, Addgene) were co-transfected into 293T cells using the jetPRIME reagent (#101000046, Polyplus-transfection), the lentiviral particles were harvested by centrifuging the supernatant of cells 48 h after transfection. Then the collected lentiviral particles were used for infecting cancer cells for 2 days in the presence of 8 ug/ml polybrene and selected by 2 ug/ml puromycin for 1 week.

CHD4 overexpression

The CHD4 cDNA clone (EX-Z1057-Lv117) and the empty vector pReceiver-Lv117 were purchased from GeneCopoeia and used for overexpressing CHD4 in thyroid cancer cells. The procedures of lentivirus production, infection and stable cell line selection were same as gene knockdown as described above.

Western blotting

Cells were lysed in the radio-immunoprecipitation assay (RIPA) buffer (#R0010, Solarbio, China) with protease inhibitor cocktail (#B14002, Bimake, China), and the western blotting analysis was performed as we described recently [33]. The anti-CHD4 (#12011S) and anti-GAPDH (#2118S) primary antibodies were purchased from Cell Signaling Technology. The HRP-linked anti-rabbit IgG (#7074S, Cell Signaling Technology) was used as the secondary antibody.

Locus specific demethylation

Targeted demethylation of *TERT* promoter region was performed by an epigenetic editing system based on CRISPR-dCas9 and Tet1 with minor modification [34]. The Flag-AviTag-dCas9-SV40NLS fragment was amplified from the pEF1a-FB-dCas9-puro plasmid (#100547, Addgene), the 3xSV40NLS-Tet1CD fragment was amplified from the Fuw-dCas9-Tet1CD plasmid (#84475, Addgene), then the two fragments were aligned and cloned into the pCDH-EF1-copGFP-T2A-Puro vector (#72263, Addgene) using the ClonExpress Ultra One Step Cloning Kit (#C115-02, Vazyme Biotech). The resulting plasmid expressing dCas9-Tet1CD fusion protein was named as pCDH-dCas9-Tet1CD. Then pCDH-dCas9-Tet1CD together with psPAX2 and pMD2.G plasmid were co-transfected into 293T cells to produce lentivirus and select stable cells after lentivirus infection. Scramble gRNA and specific gRNAs used for targeting the −209 to -420 and the −482 to -811 regions were cloned into the pSLQ1651-sgRNA(F+E)-sgGal4 plasmid (#100549, Addgene). To enhance the efficiency of targeted demethylation, six to eight gRNAs were designed for each region (Additional file 2). The lentiviral particles made by these gRNA expressing vectors were used for infecting the cells with dCas9-Tet1CD stable overexpression.

CRISPR affinity purification

The proteins binding to *TERT* promoter were identified by CRISPR affinity purification in situ of regulatory elements (CAPTURE) and mass spectrometry with minor modification [35]. The Flag-AviTag-dCas9-SV40NLS fragment was amplified from the pEF1a-FB-dCas9-puro plasmid (#100547, Addgene) and cloned into the pCDH-EF1-copGFP-T2A-Puro vector (#72263, Addgene), the resulting plasmid was named as pCDH-dCas9-puro. The BirA-V5 tag fragment was amplified from the pEF1a-BirA-V5-neo plasmid (#100548, Addgene) and cloned into the pLenti CMV/TO Hygro empty vector (#17484, Addgene), the resulting plasmid was named as pLenti-BirA-Hygro. The pCDH-dCas9-puro and pLenti-BirA-Hygro plasmids were used for generating stable cell line with dCas9 and BirA biotin simultaneous overexpression. Six gRNAs, as listed in Additional file 2, targeting near the ATG start codon as well as the scramble gRNA were cloned into the pSLQ1651-sgRNA(F+E)-sgGal4 plasmid (#100549, Addgene), and the resulting gRNA-expressing plasmids were used for making lentivirus to infect the dCas9 and BirA stably overexpressed cells.

Cells were washed twice with PBS, fixed with 1% formaldehyde (#28908, ThermoFisher) for 10 min at room temperature, and then the cell nucleus were harvested using the SimpleChIP Plus Sonication Chromatin IP Kit (#56383, Cell Signaling Technology) and incubated with *DdeI* enzyme (#R0175L, NEB) for 24 h at 37℃ in water bath. The digested products were suspended with 1X ChIP buffer and sonicated by the Bioruptor Plus device. After that, the DNA-protein complex was isolated by Dynabeads MyOne Streptavidin T1 (#65601, ThermoFisher) at 4℃ overnight, and washed 5 times with 1X ChIP buffer followed by 3 times washes with 1x high salt ChIP buffer. Finally, the beads were suspended in protein loading buffer and subjected to mass spectrometry analysis on a timsTOF Pro mass spectrometer at BGI Genomics. The resulting data were analyzed for peptides identification using Mascot Percolator program and mapped to the UniProt protein database.

Chromatin immunoprecipitation (ChIP)

The ChIP assays were performed using the SimpleChIP Plus Sonication Chromatin IP Kit (#56383, Cell Signaling Technology) according to the manufacturer's instructions. The antibodies against H3K9me3 (#13969S), H3K27me3(#9733S) and CHD4 (#12011S)

were purchased from Cell Signaling Technology, the IgG antibody (#2729, Cell Signaling Technology) was used as negative control. The final isolated DNA was amplified by qPCR with primers listed in Additional file 1.

Cell viability assay

Cells were seeded in sextuplicate on 96-well cell culture plates at a density of 1,000 cells per well and treated daily with DMSO, 5-Azacytidine or decitabine at concentrations ranging from 0.1 to 10 μm for 5 days, and the cell viability was then determined by the Cell Counting Kit-8 (#K1018, ApexBio) according to the manufacturer's instructions. The absorbance was measured at 450 nm using a spectrophotometric microplate reader. The viabilities of lentivirus infected cells were analyzed by the same procedure, instead of drug administration.

Cell apoptosis assay

Harvested cells were washed twice with cold PBS and stained with 5 μL of Pacific Blue Annexin V reagent (#640928, Biolegend) per sample for 15 min at room temperature in dark, and then incubated with 400 μL of Annexin V Binding Buffer. Lastly, the stained cells were determined by flow cytometry and analyzed by the FlowJo software.

Statistical analysis

The cell viability assays were performed twice, and each was done in sextuplicate; the other cell-based assays were repeated three times, and each was done in triplicate. All the statistical analyses were performed by GraphPad Prism (version 8.0 for Windows). Data were shown as mean ± standard deviation (SD). The differences between two groups were assessed by the Student's t-test. The half-maximal inhibitory concentrations (IC50) of the two DNA demethylating agents were calculated using the four-parameter dose-response model. All tests were 2-sided and P values < 0.05 were considered as statistically significant.

Results

Demethylating agents treatment decreased TERT expression

To explore whether DNA methylation regulates the expression of TERT in *TERT* promoter mutation-negative thyroid cancer cells, we first examined the effects of DNA demethylating agents on TERT expression. Two sets of primers were designed to cover the THOR region (Fig. 1A), the first primer sets were used for amplifying a DNA fragment that contains 29 CpG sites between −209 and −420 relative to the translational start site ATG (region I), and the second primer sets was used for amplifying the −482 to -811 region that contains 34 CpG sites (region II). After treating the TTA1 cells with two demethylating agents, 5-azacytidine and decitabine, the overall methylation level of region I was decreased from 87 to 49% and 35%, respectively; the methylation level of region II was decreased from 95 to 49% and 33%, respectively (Fig. 1B). Similarly, treatment with 5-azacytidine and decitabine induced remarkable DNA demethylation in another thyroid cancer cell line CAL-62 (Fig. 1C). Importantly, the expression of TERT was significantly decreased after 5-azacytidine or decitabine treatment in both of the two cell lines (Fig. 1D). These data suggested that *TERT* promoter methylation might positively regulates the gene's expression.

Targeted demethylation of *TERT* promoter suppressed TERT expression

To confirm the regulatory effect of promoter DNA methylation on TERT expression, we used a genomic locus-specific DNA methylation editing system based on fusion of catalytically inactive Cas9 (dCAS9) with the catalytic domain of TET1 enzyme [34] to precisely edit the CpG methylation in *TERT* promoter (Fig. 2A). Eight guide RNAs (gRNAs) were designed to induce the dCas9-TET1 fusion protein binding to the flanking sides of the −209 to -420 region. The dCas9-TET1 fusion protein was stably expressed in TTA1 cells and gRNAs were then induced into cells, two days after the pooled gRNAs delivery, the methylation level of this region was decreased from 81 to 60% (Fig. 2B) and the TERT mRNA level was correspondingly decreased (Fig. 2C). The demethylation effect on the −209 to -420 region by this locus-specific methylation editing system sustained at least 20 days (Fig. 2D) and the TERT expression was significantly lower in cells infected with pooled gRNAs targeting the −209 to -420 region than cells with the scramble gRNAs at the day 20 (Fig. 2E). Thus, demethylation of the −209 to -420 region of *TERT* promoter deceased the expression of TERT. Besides, we designed several gRNAs for demethylating the −482 to -811 region but failed to decrease the methylation level of this region.

TERT promoter hypermethylation conferred sensitivity to demethylating agents

Since the above data showed that DNA methylation of the −209 to -420 region maintained TERT expression and previous studies demonstrated that TERT inhibition suppresses the growth of thyroid cancer cells [11, 36], we next asked whether hypermethylated *TERT* promoter −209 to -420 region is a therapeutic target for thyroid cancer. To address this, we compared cells edited by the locus-specific demethylation system with the control cells, and found that demethylation of this region induced remarkable cell growth inhibition (Fig. 3A) and apoptosis (Fig. 3B). We next examined the responses of cell lines to demethylating agents. As shown in Fig. 3C, the

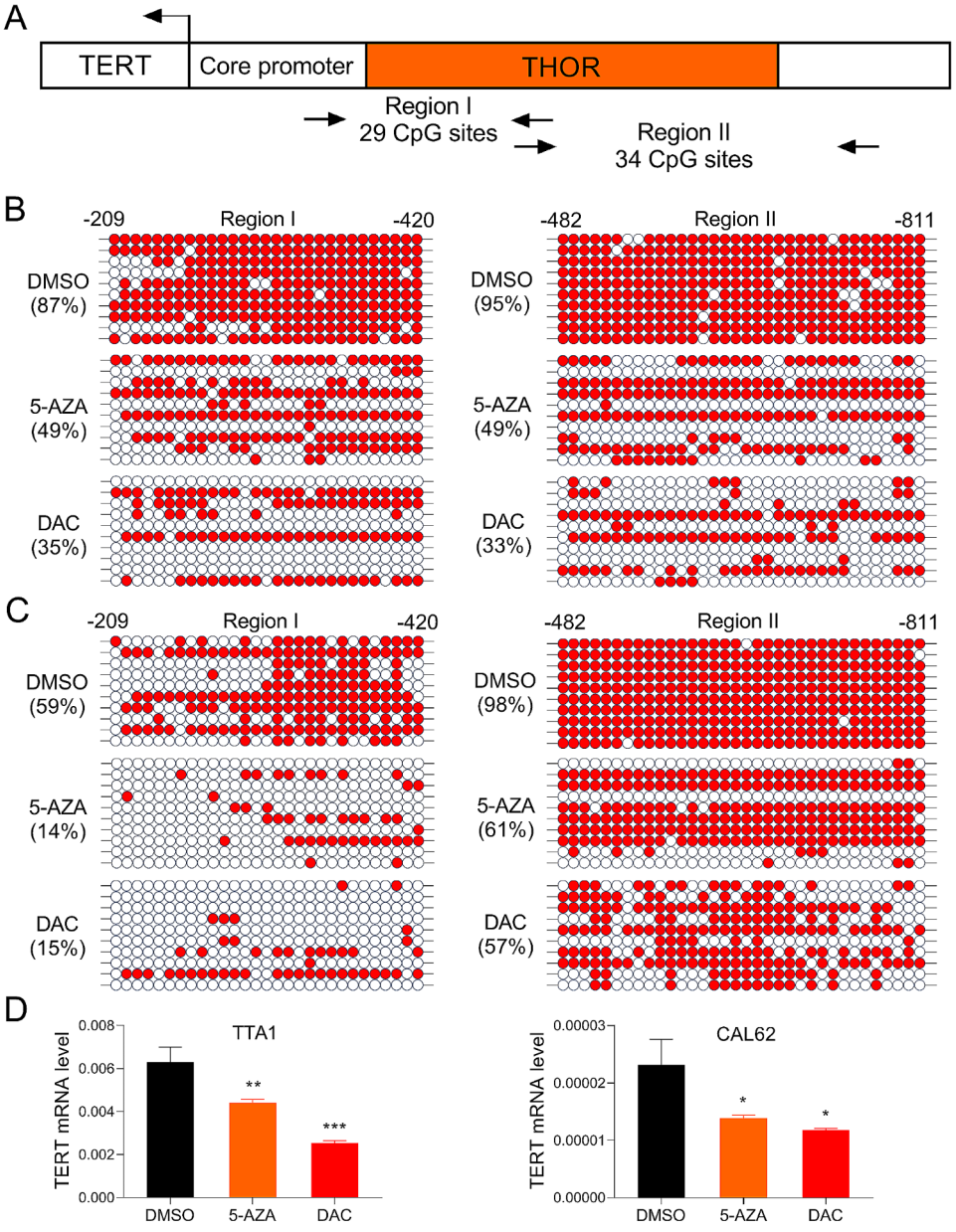

Fig. 1 Treatment cells with demethylation agents decreased expression of TERT. (**A**) Structure of promoter region of human *TERT* gene. The upstream promoter region, including the *TERT* hypermethylated oncological region (THOR), was amplified by two PCR sets for DNA methylation analysis. (**B**, **C**) Bisulfite sequencing of *TERT* upstream promoter in TTA1 (**B**) and CAL62 (**C**) cells treated with or without 1 μM of 5-azacytidine or decitabine for 5 days. (**D**) qPCR analysis of TERT mRNA expression after cells treated with 1 μM of 5-azacytidine or decitabine for 5 days. DMSO servers as control. 5-AZA, 5-azacytidine; DAC, decitabine. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

TTA1 and CAL62 cell lines were hypermethylated at the −209 to -420 region, while the MDA-T41 and WRO cell lines had relatively low methylation levels at this region. After treating these cells with 5-azacytidine, the growth of the two hypermethylated cell lines were sharply inhibited (IC50 < 1 μM), while 5-azacytidine had little effect on growth of the two hypomethylated cell lines (IC50 > 10 μM, Fig. 3D). Similar effects were observed when treating these cells with decitabine (Fig. 3E). These data suggested that DNA methylation of *TERT* promoter −209 to -420 region is a therapeutic target for thyroid cancer and hypermethylation of this region confers sensitivity to demethylating agents.

Identification of regulatory proteins at methylated *TERT* promoter

To gain insight into the molecular mechanism of how promoter DNA methylation regulates TERT expression, we adopted the CRISPR affinity purification in situ of regulatory elements (CAPTURE) approach [35] to

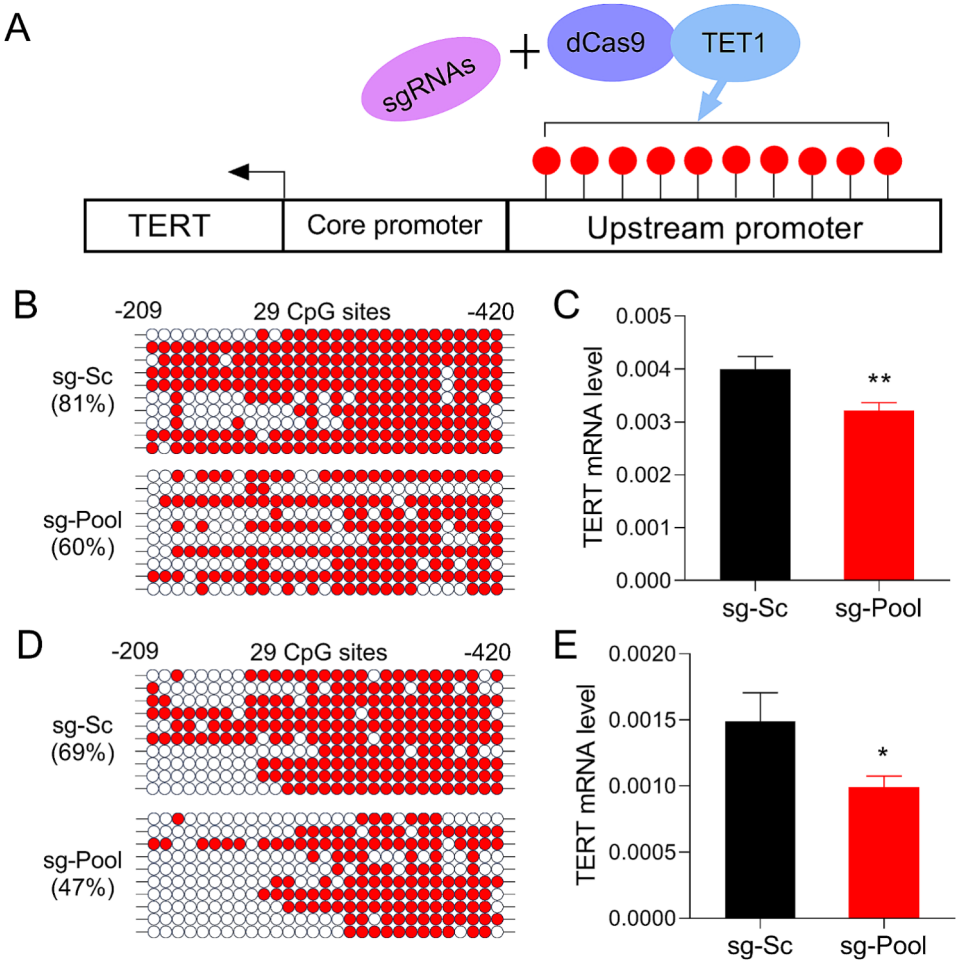

Fig. 2 Genomic locus-specific demethylation of *TERT* upstream promoter induced downregulation of TERT. (**A**) Schematic illustration of targeting *TERT* upstream promoter region by dCas9-TET1 with specific gRNAs. To enhance the targeting efficiency, eight sgRNAs were designed to induce the dCas9-TET1 fusion protein binding to the *TERT* upstream promoter. (**B**, **D**) Bisulfite sequencing of *TERT* upstream promoter (-209 to -420 region) in TTA1 cells 2 days (**B**) or 20 days (**D**) after the locus-specific demethylation stable cells were established. (**C**, **E**) qPCR analysis of TERT mRNA expression at day 2 (**C**) or day 20 (**E**) after the locus-specific demethylation stable cells were established. sg-Sc, scramble sgRNA; sg-Pool, pooled sgRNAs for targeting *TERT* upstream promoter. *$P < 0.05$, **$P < 0.01$

identify proteins binding to *TERT* promoter region in the hypermethylated TTA1 cells (Fig. 4A). After purification and mass spectrometry analysis, 311 proteins were obtained in cells infected by scramble gRNAs and 376 proteins were obtained in cells infected by gRNAs targeting *TERT* promoter, there were 133 proteins, including many DNA binding proteins, chromatin regulatory proteins and structure proteins, were identified specifically in the cells infected by gRNAs targeting *TERT* promoter (Fig. 4B, and Additional file 3). Among these proteins, we selected five for validation. As shown in Fig. 4C, in TTA1 cells, knockdown the expression of PRKDC, SMC1a, CND2 and TF3C2 increased the level of TERT, while knockdown CHD4 decreased TERT expression. Notably, there was no significant change of TERT expression after knockdown of these genes in the *TERT* hypomethylated MDA-T41 cell lines (Fig. 4C, and Additional file 4), suggesting that the regulatory effect of these genes on TERT expression is dependent on *TERT* promoter methylation.

We further examined the regulatory effect of CHD4 on *TERT* by gene overexpression (Additional file 4) and the results showed that CHD4 overexpression increased TERT expression in *TERT* hypermethylated TTA1 cells but not in the hypomethylated MDA-T41 cells (Fig. 4D). Moreover, the regulatory effect of CHD4 on TERT expression was abolished after cells treated with demethylating agent decitabine in TTA1 cells (Fig. 4E). Taken together, these data indicated that CHD4 binds to and activates the hypermethylated *TERT* promoter.

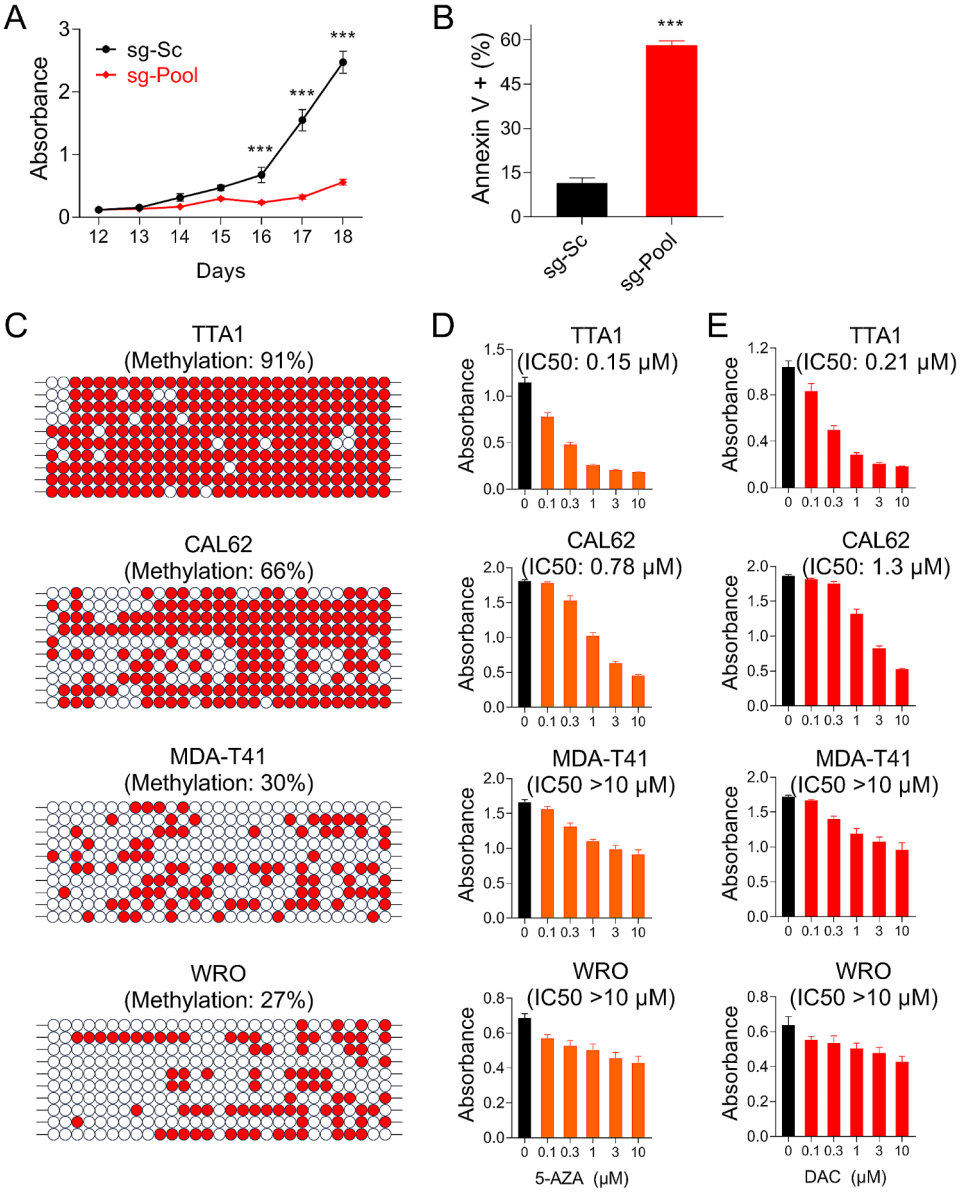

Fig. 3 The methylation status of *TERT* upstream promoter discriminated cell growth and sensitivity to demethylating agents.(**A**) Growth curves of TTA1 cells infected with gRNAs targeting *TERT* upstream promoter (sg-Pool) or the scramble gRNAs (sg-Sc). (**B**) The percentage of annexin V -positive cells after infection with sg-Pool or sg-Sc. (**C**) The basal methylation status of *TERT* upstream promoter (-209 to -420 region) in four thyroid cancer cell lines. (**D**, **E**) Growth curves of thyroid cancer cells treated with 5-azacytidine (**D**) or decitabine (**E**) at indicated concentrations for 5 days. 5-AZA, 5-azacytidine; DAC, decitabine. ***$P < 0.001$

CHD4 suppressed the inhibitory histone modifications at *TERT* promoter

Lastly, we took efforts to explore how CHD4 regulates TERT expression. The ChIP assay revealed that CHD4 antibody -precipitated DNA fragments were enriched at the −213 to -466 region of *TERT* promoter (Fig. 5A), and the methylation level of this region was not remarkably changed after *CHD4* knockdown (Fig. 5B), indicating that CHD4 was not involved in the de novo methylation or demethylation of *TERT* promoter. Since histone modification is frequently couples with DNA methylation to regulates gene expression, we next assessed whether CHD4 affects the histone modification at *TERT* promoter, and the results showed that CHD4 knockdown significantly increased the enrichment of two inhibitory markers (H3K9me3 and H3K27me3) at *TERT* promoter (Fig. 5C). Moreover, the demethylating agent decitabine inhibited the binding of CHD4 to *TERT* promoter (Fig. 5D) and increased the enrichment of H3K9me3 and H3K27me3 at *TERT* promoter (Fig. 5E). Taken together, these data suggested that CHD4 regulates TERT

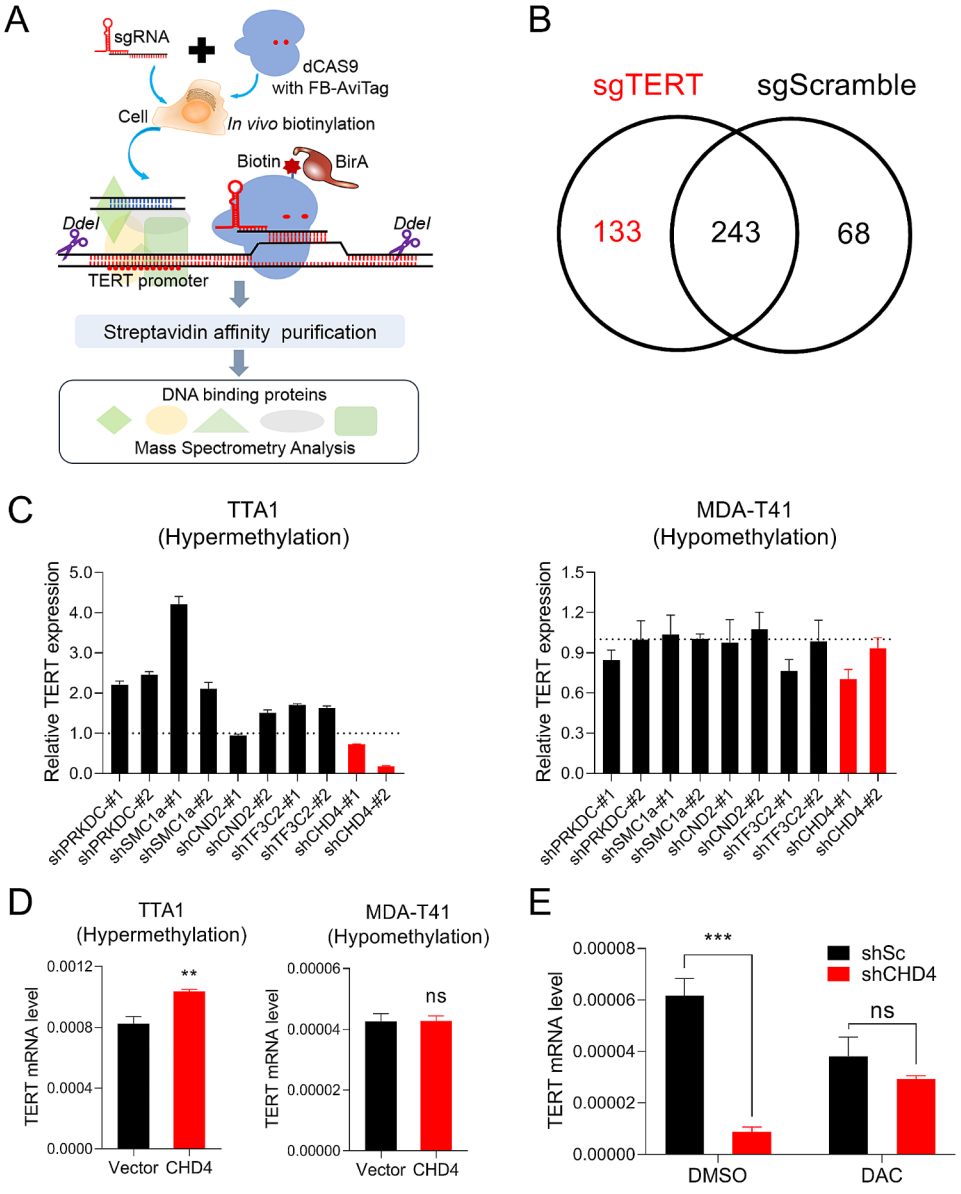

Fig. 4 CHD4 regulated TERT expression. (**A**) Schematic of biotinylated dCas9-mediated capture of proteins interacted with *TERT* promoter. (**B**) The number of proteins captured by gRNA targeting *TERT* promoter (sgTERT) or scramble gRNAs (sgScramble) in TTA1 cells. (**C**) qPCR analysis of relative TERT expression after knockdown of potential regulatory genes in TTA1 and MDA-T41 cells. (**D**) qPCR analysis of TERT mRNA expression after CHD4 overexpression. (**E**) qPCR analysis of TERT mRNA expression after CHD4 knockdown in TTA1 cells treated with or without 1 μM of decitabine for 5 days. DAC, decitabine. $^{**}P < 0.01$, $^{***}P < 0.001$; ns, not significant

expression by affecting histone modifications at hypermethylated *TERT* promoter region.

Discussion

High expression of TERT has been linked to poor prognosis of papillary thyroid cancer, the most common histological subtype of thyroid cancer [37]. We and other groups previous demonstrated that TERT overexpression in thyroid cancer was partially attributed to the two hot-spot mutations in the core promoter region of *TERT*, multiple members of the ETS transcriptional factors, including ETV1, ETV4, ETV5, and the GABPA/GABPB complex selectively binds to and activates the mutant *TERT* promoter [10–13]. And a strong association between *TERT* promoter mutation and aggressive behaviors and poor prognosis of thyroid cancer have been established by a large number of studies [9, 38–40].

Similar to *TERT* promoter mutation, hypermethylation of *TERT* promoter has been observed in patients with thyroid cancer and several cohort studies revealed that thyroid cancer patients with hypermethylated *TERT* promoter had more aggressive characteristics and worse

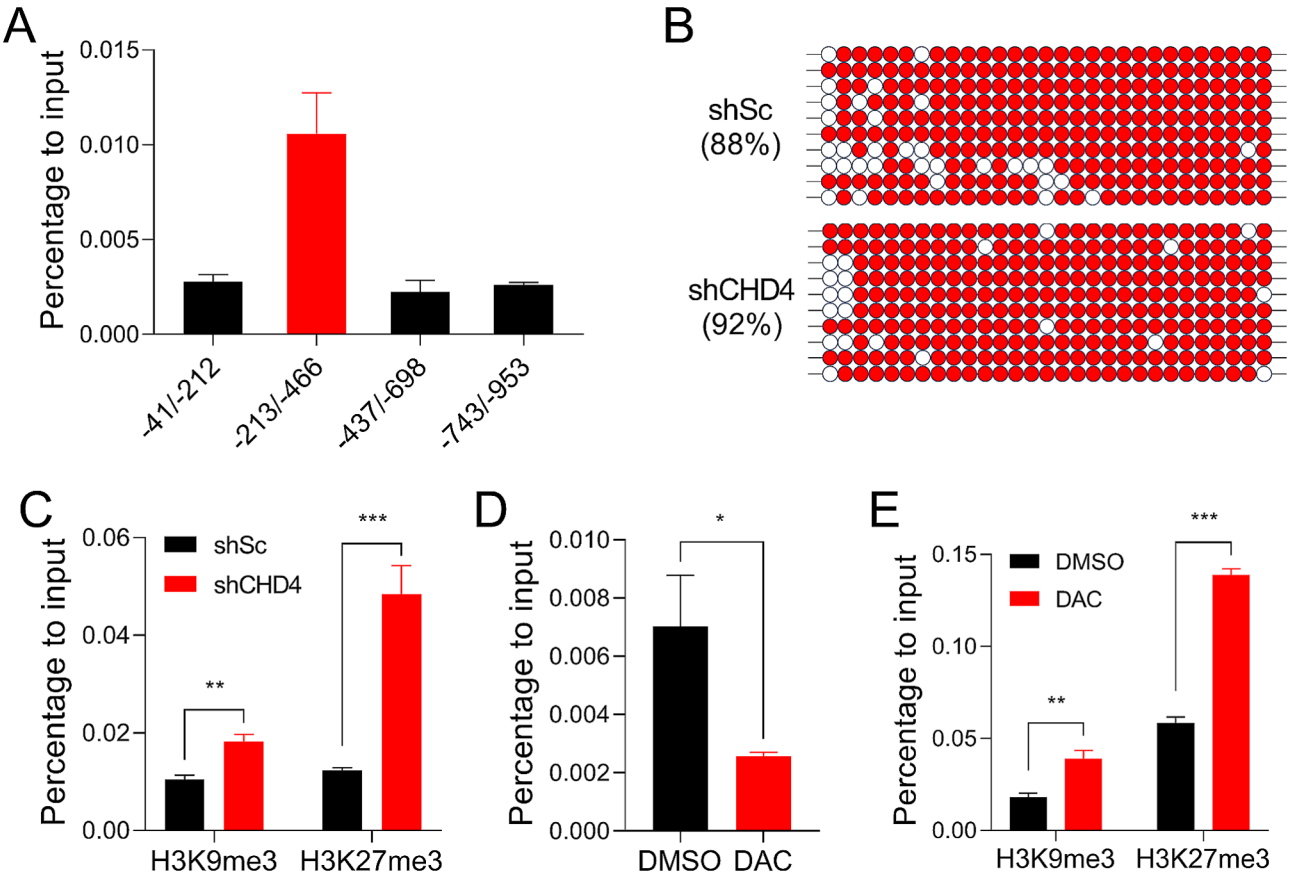

Fig. 5 CHD4 suppressed enrichment of inhibitory histone modifications at *TERT* promoter region. (**A**) Chromatin immunoprecipitation (ChIP) assay for CHD4 occupancy at different regions of *TERT* promoter in TTA1 cells. (**B**) Bisulfite sequencing of *TERT* upstream promoter (-209 to -420 region) in TTA1 cells with or without CHD4 stable knockdown. (**C**) ChIP assay for H3K9me3 and H3K27me3 occupancy at *TERT* promoter in TTA1 cells with or without CHD4 knockdown. (**D**) ChIP assay for CHD4 occupancy at *TERT* promoter in TTA1 cells treated with or without 1 μM of decitabine for 5 days. (**E**) ChIP assay for H3K9me3 and H3K27me3 occupancy at *TERT* promoter in TTA1 cells treated with or without 1 μM of decitabine for 5 days. DAC, decitabine. **P* < 0.05, ***P* < 0.01, ****P* < 0.001

clinical outcomes than patients with hypomethylated *TERT* promoter [22–26]. However, the regulatory role of promoter DNA methylation on TERT expression in thyroid cancer has not been well understood [28]. A pioneer study detected the methylation status of the region −662 to +174 relative to the transcription start site of TERT in thyroid cancer cell lines and normal thyroid tissue samples, and found that the core promoter region was hypomethylated and the upstream portions of *TERT* promoter was hypermethylated in thyroid cancer [41]. Importantly, putative binding sites of transcription activator MYC and homebox protein goosecoid (GSC) was identified in the upstream *TERT* promoter [41], suggesting a potential regulatory role of this region in TERT expression.

In this study, by treating thyroid cancer cell lines with DNA methyltransferase (DNMT) inhibitors 5-azacytidine and decitabine, we found that the methylation levels of the upstream region −209 to -811 relative to the translational start site ATG were significantly decreased, and importantly, the expressions of TERT were correspondingly decreased in both the two cell lines we tested. Consistently, *TERT* transcription was strongly repressed after treatment with DNMT inhibitors in cell lines derived from other cancer types [42–44]. We further applied a CRISPR-dCas9 based DNA demethylation system to specifically suppress the methylation level of the upstream *TERT* promoter, and the successful locus specific demethylation of the region −209 to -420 induced downregulation of TERT transcription. These findings in the current study demonstrated that DNA methylation in the upstream *TERT* promoter maintains TERT expression in thyroid cancer. Moreover, we observed that targeted demethylation of the region −209 to -420 sharply inhibited cell growth by inducing apoptosis. This is consistent with previous findings that genetical or pharmacological TERT inhibition induced apoptosis in cancer cells and targeted THOR demethylation impaired the development of histologic cancer phenotype in a breast cancer xenograft model [21, 32, 45]. Together, these findings strongly indicated that hypermethylation of *TERT*

upstream promoter is a therapeutic target for thyroid cancer.

The two classic DNA demethylating agents 5-azacytidine and decitabine has been proved by FDA between 2004 and 2006 for treating patients with hematological malignancies. However, the cytotoxic side effects limited their clinical application for solid tumors. Strikingly, Park et al. revealed that TERT overexpression increased DNMT1 expression and low dose of decitabine resulted in significant response in gliomas with high expression of TERT and DNMT1 [46]. Here, in this study we found that the hypermethylation of the upstream *TERT* promoter conferred sensitivity to 5-azacytidine and decitabine in thyroid cancer cells. Although our finding needs to be confirmed in more cell lines, these data indicated that high expression of TERT or hypermethylation in the upstream *TERT* promoter have the potential to be effective biomarkers for stratifying patients to maximize the therapeutic efficacy of DNA demethylating agents in solid tumors.

Although the methylation of *TERT* promoter in TERT-expressing telomerase positive cancer tissue samples or cell lines had been initially reported over twenty years [47–49], and the association between promoter DNA methylation and the gene's expression is still not fully understood. It has been demonstrated that absence of methylation in the core promoter region associated with active chromatin marks binding and TERT expression [50–52]. By analyzing the interplay between *TERT* promoter mutation, methylation and expression at the allele level, recent studies revealed that hypomethylation of *TERT* distal promoter is specific to active alleles in cells harboring *TERT* promoter mutation [29–31], while transcriptionally active alleles exhibit hypermethylation in the THOR or distal promoter region in *TERT* wild-type cells [53, 54]. It should be noted that all the cell lines used in the current study were *TERT* wild-type, and consistent with the above-mentioned studies, our results confirmed a positively regulatory role of hypermethylation of *TERT* upstream promoter in TERT expression in *TERT* wide-type cells.

Mechanismly, we identified a number of regulatory proteins bound at the methylated *TERT* promoter and verified the regulation of PRKDC, SMC1a, CND2, TF3C2 and CHD4 on TERT expression. As a core subunit of the nucleosome remodeling and deacetylase complex, CHD4 plays important roles in chromatin remodeling and epigenetic transcriptional regulation [55]. It maintains DNA hypermethylation-associated transcriptional silencing of tumor suppressor genes by recruiting repressive chromatin proteins and involves in the progression of colorectal cancer [56]. Recently, overexpression and genetic variants of CHD4 were reported in patients with advanced papillary thyroid cancer [57, 58], suggesting an oncogenic role of CHD4 in thyroid cancer. Here we found that CHD4 did not affect the methylation level of *TERT* promoter but bound to the hypermethylated *TERT* upstream promoter and activates its expression. The interplay between DNA and histone methylation in gene regulation has been well established in the past three decades, and some inhibitory histone modifications, such as H3K9me3 and H3K27me3, were representative markers of heterochromatin, which associated with gene inactivation [59, 60]. In this study we found that CHD4 suppressed the enrichment of H3K9me3 and H3K27me3 at hypermethylated *TERT* promoter, suggesting that chromatin remodeling was involved in promoter DNA methylation mediated TERT regulation. In addition to thyroid cancer, *TERT* upstream promoter methylation was indicated to corelated with increased expression of TERT in several other types of cancer [17–21, 27]; therefore, it is worth to test whether CHD4 and chromatin remodeling participate in TERT regulation in these cancer types. Moreover, the molecular mechanism of how other hypermethylated *TERT* promoter binding proteins regulates the gene's expression needs to be explored further.

Conclusion

In summary, our results show a positive regulatory effect of hypermethylation of *TERT* upstream promoter on the gene's expression in *TERT* promoter mutation negative thyroid cancer. The chromatin remodeling protein CHD4 binds to the hypermethylated *TERT* promoter, suppresses the inhibitory histone modification, and leads to TERT upregulation. Locus-specific demethylation for *TERT* upstream promoter region induces cell apoptosis and growth inhibition, and hypermethylation of *TERT* upstream promoter confers sensitivity to DNMT inhibitors, suggesting it could be a therapeutic target for *TERT* promoter wild-type thyroid cancers.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12935-024-03459-2.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Acknowledgements

We thank all the other members of the Liu's laboratory for their technical and scientific assistance.

Author contributions

RL and SL designed the research; SL, GH, YC, YS and QT performed the research and collected the original data; SL, YC and RL analyzed the data; SL and RL wrote the manuscript, with inputs from all authors. Each author read and approved the final manuscript.

Funding
This study was supported by grants from the National Natural Science Foundation of China (No. 82222051 and No. 82072952), and the Fundamental Research Funds for the Central Universities, Sun Yat-sen University (No. 22ykqb05).

Data availability
All the data presented in this study are included in the article and supplementary materials.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Institute of Precision Medicine, The First Affiliated Hospital, Sun Yat-sen University, No. 58, Zhongshan Second Road, Guangzhou, Guangdong 510080, China

Received: 26 January 2024 / Accepted: 25 July 2024
Published online: 03 August 2024

References

1. Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I, Jemal A. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2024;74(3):229–63.
2. Chen M, Xue J, Sang Y, Jiang W, He W, Hong S, Lv W, Xiao H, Liu R. Highly sensitive droplet digital PCR for detection of RET fusion in papillary thyroid cancer. BMC Cancer. 2023;23(1):363.
3. Landa I, Cabanillas ME. Genomic alterations in thyroid cancer: biological and clinical insights. Nat Rev Endocrinol. 2024;20(2):93–110.
4. Zeng PYF, Prokopec SD, Lai SY, Pinto N, Chan-Seng-Yue MA, Clifton-Bligh R, Williams MD, Howlett CJ, Plantinga P, Cecchini MJ, et al. The genomic and evolutionary landscapes of anaplastic thyroid carcinoma. Cell Rep. 2024;43(3):113826.
5. Xiao X, Chen M, Sang Y, Xue J, Jiang K, Chen Y, Zhang L, Yu S, Lv W, Li Y, et al. Methylation-mediated silencing of ATF3 promotes thyroid Cancer progression by regulating prognostic genes in the MAPK and PI3K/AKT pathways. Thyroid. 2023;33(12):1441–54.
6. Zafon C, Gil J, Perez-Gonzalez B, Jorda M. DNA methylation in thyroid cancer. Endocr Relat Cancer. 2019;26(7):R415–39.
7. Low KC, Tergaonkar V. Telomerase: central regulator of all of the hallmarks of cancer. Trends Biochem Sci. 2013;38(9):426–34.
8. Liu M, Zhang Y, Jian Y, Gu L, Zhang D, Zhou H, Wang Y, Xu ZX. The regulations of telomerase reverse transcriptase (TERT) in cancer. Cell Death Dis. 2024;15(1):90.
9. Liu R, Xing M. TERT promoter mutations in thyroid cancer. Endocr Relat Cancer. 2016;23(3):R143–155.
10. Bullock M, Lim G, Zhu Y, Aberg H, Kurdyukov S, Clifton-Bligh R. ETS factor ETV5 activates the mutant telomerase reverse transcriptase promoter in thyroid Cancer. Thyroid. 2019;29(11):1623–33.
11. Liu R, Zhang T, Zhu G, Xing M. Regulation of mutant TERT by BRAF V600E/MAP kinase pathway through FOS/GABP in human cancer. Nat Commun. 2018;9(1):579.
12. Song YS, Yoo SK, Kim HH, Jung G, Oh AR, Cha JY, Kim SJ, Cho SW, Lee KE, Seo JS, et al. Interaction of BRAF-induced ETS factors with mutant TERT promoter in papillary thyroid cancer. Endocr Relat Cancer. 2019;26(6):629–41.
13. Thornton CEM, Hao J, Tamarapu PP, Landa I. Multiple ETS factors participate in the Transcriptional Control of TERT Mutant promoter in thyroid cancers. Cancers (Basel). 2022;14(2):357.
14. Yuan X, Larsson C, Xu D. Mechanisms underlying the activation of TERT transcription and telomerase activity in human cancer: old actors and new players. Oncogene. 2019;38(34):6172–83.
15. Lee DD, Leao R, Komosa M, Gallo M, Zhang CH, Lipman T, Remke M, Heidari A, Nunes NM, Apolonio JD, et al. DNA hypermethylation within TERT promoter upregulates TERT expression in cancer. J Clin Invest. 2019;129(1):223–9.
16. Barthel FP, Wei W, Tang M, Martinez-Ledesma E, Hu X, Amin SB, Akdemir KC, Seth S, Song X, Wang Q, et al. Systematic analysis of telomere length and somatic alterations in 31 cancer types. Nat Genet. 2017;49(3):349–57.
17. Castelo-Branco P, Choufani S, Mack S, Gallagher D, Zhang C, Lipman T, Zhukova N, Walker EJ, Martin D, Merino D, et al. Methylation of the TERT promoter and risk stratification of childhood brain tumours: an integrative genomic and molecular study. Lancet Oncol. 2013;14(6):534–42.
18. Castelo-Branco P, Leao R, Lipman T, Campbell B, Lee D, Price A, Zhang C, Heidari A, Stephens D, Boerno S, et al. A cancer specific hypermethylation signature of the TERT promoter predicts biochemical relapse in prostate cancer: a retrospective cohort study. Oncotarget. 2016;7(36):57726–36.
19. Faleiro I, Apolonio JD, Price AJ, De Mello RA, Roberto VP, Tabori U, Castelo-Branco P. The TERT hypermethylated oncologic region predicts recurrence and survival in pancreatic cancer. Future Oncol. 2017;13(23):2045–51.
20. Leao R, Lee D, Figueiredo A, Hermanns T, Wild P, Komosa M, Lau I, Mistry M, Nunes NM, Price AJ, et al. Combined genetic and epigenetic alterations of the TERT promoter affect clinical and biological behavior of bladder cancer. Int J Cancer. 2019;144(7):1676–84.
21. Apolonio JD, Dias JS, Fernandes MT, Komosa M, Lipman T, Zhang CH, Leao R, Lee D, Nunes NM, Maia AT, et al. THOR is a targetable epigenetic biomarker with clinical implications in breast cancer. Clin Epigenetics. 2022;14(1):178.
22. Wang N, Kjellin H, Sofiadis A, Fotouhi O, Juhlin CC, Backdahl M, Zedenius J, Xu D, Lehtio J, Larsson C. Genetic and epigenetic background and protein expression profiles in relation to telomerase activation in medullary thyroid carcinoma. Oncotarget. 2016;7(16):21332–46.
23. Li JJ, Zheng PCJ, Wang YZ. The correlations between DNA methylation and polymorphisms in the promoter region of the human telomerase reverse transcriptase (hTERT) gene with postoperative recurrence in patients with thyroid carcinoma (TC). World J Surg Oncol. 2017;15(1):114.
24. Paulsson JO, Mu N, Shabo I, Wang N, Zedenius J, Larsson C, Juhlin CC. TERT aberrancies: a screening tool for malignancy in follicular thyroid tumours. Endocr Relat Cancer. 2018;25(7):723–33.
25. Montero-Conde C, Leandro-Garcia LJ, Martinez-Montes AM, Martinez P, Moya FJ, Leton R, Gil E, Martinez-Puente N, Guadalix S, Curras-Freixes M, et al. Comprehensive molecular analysis of immortalization hallmarks in thyroid cancer reveals new prognostic markers. Clin Transl Med. 2022;12(8):e1001.
26. Li S, Xue J, Jiang K, Chen Y, Zhu L, Liu R. TERT promoter methylation is associated with high expression of TERT and poor prognosis in papillary thyroid cancer. Front Oncol. 2024;14:1325345.
27. Lee DD, Komosa M, Nunes NM, Tabori U. DNA methylation of the TERT promoter and its impact on human cancer. Curr Opin Genet Dev. 2020;60:17–24.
28. McKelvey BA, Umbricht CB, Zeiger MA. Telomerase Reverse transcriptase (TERT) regulation in thyroid Cancer: a review. Front Endocrinol (Lausanne). 2020;11:485.
29. Stern JL, Paucek RD, Huang FW, Ghandi M, Nwumeh R, Costello JC, Cech TR. Allele-specific DNA methylation and its interplay with repressive histone marks at promoter-mutant TERT genes. Cell Rep. 2017;21(13):3700–7.
30. Esopi D, Graham MK, Brosnan-Cashman JA, Meyers J, Vaghasia A, Gupta A, Kumar B, Haffner MC, Heaphy CM, De Marzo AM, et al. Pervasive promoter hypermethylation of silenced TERT alleles in human cancers. Cell Oncol (Dordr). 2020;43(5):847–61.
31. McKelvey BA, Gilpatrick T, Wang Y, Timp W, Umbricht CB, Zeiger MA. Characterization of allele-specific regulation of Telomerase Reverse transcriptase in promoter mutant thyroid Cancer cell lines. Thyroid. 2020;30(10):1470–81.
32. Liu R, Tan J, Shen X, Jiang K, Wang C, Zhu G, Xing M. Therapeutic targeting of FOS in mutant TERT cancers through removing TERT suppression of apoptosis via regulating survivin and TRAIL-R2. Proc Natl Acad Sci U S A. 2021;118(11):e2022779118.
33. Chen Y, Sang Y, Li S, Xue J, Chen M, Hong S, Lv W, Sehgal K, Xiao H, Liu R. The ERK inhibitor GDC-0994 selectively inhibits growth of BRAF mutant cancer cells. Transl Oncol. 2024;45:101991.
34. Liu XS, Wu H, Ji X, Stelzer Y, Wu X, Czauderna S, Shu J, Dadon D, Young RA, Jaenisch R. Editing DNA methylation in the mammalian genome. Cell. 2016;167(1):233–47.

35. Liu X, Zhang Y, Chen Y, Li M, Zhou F, Li K, Cao H, Ni M, Liu Y, Gu Z, et al. In situ capture of chromatin interactions by biotinylated dCas9. Cell. 2017;170(5):1028–43.
36. Maggisano V, Celano M, Lombardo GE, Lepore SM, Sponziello M, Rosignolo F, Verrienti A, Baldan F, Puxeddu E, Durante C, et al. Silencing of hTERT blocks growth and migration of anaplastic thyroid cancer cells. Mol Cell Endocrinol. 2017;448:34–40.
37. Tanaka A, Matsuse M, Saenko V, Nakao T, Yamanouchi K, Sakimura C, Yano H, Nishihara E, Hirokawa M, Suzuki K, et al. TERT mRNA expression as a novel prognostic marker in papillary thyroid carcinomas. Thyroid. 2019;29(8):1105–14.
38. Melo M, da Rocha AG, Vinagre J, Batista R, Peixoto J, Tavares C, Celestino R, Almeida A, Salgado C, Eloy C, et al. TERT promoter mutations are a major indicator of poor outcome in differentiated thyroid carcinomas. J Clin Endocrinol Metab. 2014;99(5):E754–765.
39. Kim TH, Kim YE, Ahn S, Kim JY, Ki CS, Oh YL, Kim K, Yun JW, Park WY, Choe JH, et al. TERT promoter mutations and long-term survival in patients with thyroid cancer. Endocr Relat Cancer. 2016;23(10):813–23.
40. Park J, Lee S, Kim K, Park H, Ki CS, Oh YL, Shin JH, Kim JS, Kim SW, Chung JH, et al. TERT promoter mutations and the 8th Edition TNM classification in Predicting the survival of thyroid Cancer patients. Cancers (Basel). 2021;13(4):648.
41. Avin BA, Wang Y, Gilpatrick T, Workman RE, Lee I, Timp W, Umbricht CB, Zeiger MA. Characterization of human telomerase reverse transcriptase promoter methylation and transcription factor binding in differentiated thyroid cancer cell lines. Genes Chromosomes Cancer. 2019;58(8):530–40.
42. Guilleret I, Benhattar J. Demethylation of the human telomerase catalytic subunit (hTERT) gene promoter reduced hTERT expression and telomerase activity and shortened telomeres. Exp Cell Res. 2003;289(2):326–34.
43. Kumari A, Srinivasan R, Wig JD. Effect of c-MYC and E2F1 gene silencing and of 5-azacytidine treatment on telomerase activity in pancreatic cancer-derived cell lines. Pancreatology. 2009;9(4):360–8.
44. Pettigrew KA, Armstrong RN, Colyer HA, Zhang SD, Rea IM, Jones RE, Baird DM, Mills KI. Differential TERT promoter methylation and response to 5-aza-2'-deoxycytidine in acute myeloid leukemia cell lines: TERT expression, telomerase activity, telomere length, and cell death. Genes Chromosomes Cancer. 2012;51(8):768–80.
45. Cao Y, Li H, Deb S, Liu JP. TERT regulates cell survival independent of telomerase enzymatic activity. Oncogene. 2002;21(20):3130–8.
46. Park JW, Sahm F, Steffl B, Arrillaga-Romany I, Cahill D, Monje M, Herold-Mende C, Wick W, Turcan S. TERT and DNMT1 expression predict sensitivity to decitabine in gliomas. Neuro Oncol. 2021;23(1):76–87.
47. Devereux TR, Horikawa I, Anna CH, Annab LA, Afshari CA, Barrett JC. DNA methylation analysis of the promoter region of the human telomerase reverse transcriptase (hTERT) gene. Cancer Res. 1999;59(24):6087–90.
48. Dessain SK, Yu H, Reddel RR, Beijersbergen RL, Weinberg RA. Methylation of the human telomerase gene CpG island. Cancer Res. 2000;60(3):537–41.
49. Guilleret I, Yan P, Grange F, Braunschweig R, Bosman FT, Benhattar J. Hypermethylation of the human telomerase catalytic subunit (hTERT) gene correlates with telomerase activity. Int J Cancer. 2002;101(4):335–41.
50. Zinn RL, Pruitt K, Eguchi S, Baylin SB, Herman JG. hTERT is expressed in cancer cell lines despite promoter DNA methylation by preservation of unmethylated DNA and active chromatin around the transcription start site. Cancer Res. 2007;67(1):194–201.
51. Renaud S, Loukinov D, Abdullaev Z, Guilleret I, Bosman FT, Lobanenkov V, Benhattar J. Dual role of DNA methylation inside and outside of CTCF-binding regions in the transcriptional regulation of the telomerase hTERT gene. Nucleic Acids Res. 2007;35(4):1245–56.
52. Rowland TJ, Bonham AJ, Cech TR. Allele-specific proximal promoter hypomethylation of the telomerase reverse transcriptase gene (TERT) associates with TERT expression in multiple cancers. Mol Oncol. 2020;14(10):2358–74.
53. Lee DD, Komosa M, Sudhaman S, Leao R, Zhang CH, Apolonio JD, Hermanns T, Wild PJ, Klocker H, Nassiri F, et al. Dual role of allele-specific DNA hypermethylation within the TERT promoter in cancer. J Clin Invest. 2021;131(21):e146915.
54. Lee S, Chang TC, Schreiner P, Fan Y, Agarwal N, Owens C, Dummer R, Kirkwood JM, Barnhill RL, Theodorescu D, et al. Targeted Long-Read Bisulfite sequencing identifies differences in the TERT promoter methylation profiles between TERT Wild-Type and TERT Mutant Cancer cells. Cancers (Basel). 2022;14(16):4018.
55. Denslow SA, Wade PA. The human Mi-2/NuRD complex and gene regulation. Oncogene. 2007;26(37):5433–8.
56. Xia L, Huang W, Bellani M, Seidman MM, Wu K, Fan D, Nie Y, Cai Y, Zhang YW, Yu LR, et al. CHD4 has oncogenic functions in initiating and maintaining epigenetic suppression of multiple tumor suppressor genes. Cancer Cell. 2017;31(5):653–68.
57. Pratheeshkumar P, Siraj AK, Divya SP, Parvathareddy SK, Alobaisi K, Al-Sobhi SS, Al-Dayel F, Al-Kuraya KS. CHD4 predicts aggressiveness in PTC patients and promotes Cancer stemness and EMT in PTC cells. Int J Mol Sci. 2021;22(2):504.
58. Masoodi T, Siraj AK, Siraj S, Azam S, Qadri Z, Albalawy WN, Parvathareddy SK, Al-Sobhi SS, Al-Dayel F, Alkuraya FS, et al. Whole-exome sequencing of Matched Primary and metastatic papillary thyroid Cancer. Thyroid. 2020;30(1):42–56.
59. Michalak EM, Burr ML, Bannister AJ, Dawson MA. The roles of DNA, RNA and histone methylation in ageing and cancer. Nat Rev Mol Cell Biol. 2019;20(10):573–89.
60. Li Y, Chen X, Lu C. The interplay between DNA and histone methylation: molecular mechanisms and disease implications. EMBO Rep. 2021;22(5):e51803.

Publisher's Note