



Promoter CpG methylation inhibits Krüppel-like factor 2 (KLF2)-Mediated repression of hTERT gene expression in human T-cells

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ABSTRACT

Constitutive expression of human telomerase reverse transcriptase (hTERT) with DNA methylation of its promoter is a common phenomenon in tumor cells. We recently found that the transcriptional factor Krüppel-like factor 2 (KLF2) binds to the CpG sequences in the hTERT promoter and inhibits hTERT gene expression in normal resting T-cells. The human T-cell line Kit 225 in the resting phase induced by the deprivation of interleukin (IL)-2 showed no decrease in the expression of hTERT, despite the high expression of KLF2. To elucidate the mechanisms of deregulation of hTERT expression in T-cells, we examined the relationship between DNA methylation and KLF2 binding to the hTERT promoter. The hTERT promoter was methylated in Kit 225 cells, resulting in the inhibition of the binding of KLF2 to the promoter. DNA demethylation by the reagent Zebularine recovered KLF2 binding to the hTERT promoter, followed by the downregulation of its gene expression. These findings indicate that the repressive effect of KLF2 on hTERT gene expression is abolished by DNA methylation in T-cell lines.

1. Introduction

Telomere, a structure of the chromosomal terminus, compensates for the end-replication problem. Embryonic stem cells and germline cells maintain the telomere length, because these cells express human telomerase reverse transcriptase (hTERT), which regulates telomerase activity. hTERT expression is very low in most somatic cells to limit the capacity for cell division [1,2]. Normal lymphocytes exceptionally express hTERT after immune reactions to promote cellular expansion, while hTERT expression is strictly repressed in lymphocytes at the resting phase [3–5]. We recently identified the transcription factor Krüppel-like factor 2 (KLF2) as a transcriptional repressor of the hTERT gene in human normal resting T-cells [6]. KLF2 is a key molecule involved in cellular quiescence and its expression is inversely correlated with hTERT expression [6–8].

Telomerase activity is considered a hallmark of cancer progression, owing to the constitutive expression of hTERT in approximately 80% tumors [9,10]. A considerable number of genes are thought to be methylated at CpG sequences and known to be silenced in cancer cells

[11]. Similar to these genes, the hTERT gene promoter is methylated, while the hTERT gene is actively transcribed in cancer cells [12–14]. The effects of DNA methylation on hTERT expression remain to be elucidated.

We hypothesized that KLF2-mediated regulation of hTERT gene expression is out of control in the human leukemic T-cell line. Our results demonstrate that hTERT promoter was methylated in Kit 225 cells and that KLF2 binding to the hTERT promoter was hindered by DNA methylation, leading to the induction of hTERT expression. Our findings highlight the mechanism underlying the regulation of hTERT expression by promoter methylation.

2. Materials and methods

2.1. Materials

Anti-KLF2 antibody (AB4137) was purchased from Merck Millipore (Darmstadt, Germany). Anti-KLF2 (sc-28675), anti-c-Myc (sc-764), and anti- β -tubulin (sc-9104) antibodies and normal rabbit IgG (sc-2017)

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were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Zebularine was obtained from Wako (Osaka, Japan). The reporter plasmids carrying hTERT promoter fragments, pTERT(-281)-L and pGL3-TERT/(+9-+30) × 3 were described elsewhere [6,16]. Small interfering RNAs (siRNAs) for KLF2 (10620318-343842F07, 1062318-343842F08, and 10620318-343842E11) and control siRNA were purchased from Thermo Fisher SCIENTIFIC (Waltham, MA, USA).

2.2. Cells and cell culture

Peripheral blood samples from healthy donors were obtained with approval from the Internal Review Committees of Tokyo Medical and Dental University (ethical code number: 197). We enrolled participants into the study after obtaining written informed consent. All experiments were performed in accordance with the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ, USA). Normal T-cells were separated from PBMCs with Pan T cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). The interleukin (IL)-2-dependent human T-cell line Kit 225 (kindly provided by T. Hori), which is derived from a patient with T-cell chronic lymphocytic leukemia was cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS) and 1 nM of IL-2 (Shionogi, Osaka, Japan) and was passaged less than 2 months [15]. Cells were authenticated by short tandem repeat (STR) and the cell surface markers (CD4 and CD25) analyses in addition to Mycoplasma rRNA gene detection by in-house nested PCR, which were performed in 2019. DNA demethylation was induced by the addition of 50 μM Zebularine.

2.3. Transfection and reporter assay

The reporter plasmids were introduced into cells by the DEAE-dextran method, as previously described [17]. Cells were cultured for 40 h with or without IL-2, and luciferase activity was measured using Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's protocol. siRNA was introduced using Neon Transfection System (Thermo Fisher SCIENTIFIC). Electroporation for Kit 225 cells was performed at 1200 V (voltage) for 10 ms (width) in pulse number 3.

2.4. RNA isolation and reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using Isogen (Nippon Gene, Toyama, Japan) and AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) according to the suppliers' protocols. First-strand cDNA was synthesized using the 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Applied Science, Indianapolis, IN, USA). Quantification of hTERT mRNA was performed using LightCycler FastStart DNA Master SYBR Green I with LightCycler (Roche Applied Science) according to the manufacturer's protocol. The PCR primers sequences designed by Lasergene software (DNASTAR, Madison, WI, USA) were shown in Table 1. The primers for 18 S rRNA were obtained from TaKaRa (Shiga, Japan).

2.5. Immunoblotting analysis

Cells were lysed in the RIPA buffer containing 150 mM sodium

chloride (NaCl), 50 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% sodium dodecyl sulfate (SDS). Cell lysates containing 50 μg of protein were electrophoresed on 10% SDS-polyacrylamide gels and blotted onto Immobilon-P membranes (Merck Millipore). The membranes were incubated with primary antibodies for KLF2 and β-tubulin (1:1000) overnight at 4 °C, and were treated with horseradish peroxidase-conjugated secondary antibodies (1:5,000, GE Healthcare) for 60 min at 25 °C. Detection was performed with Luminata Crescendo Western HRP Substrate (Merck Millipore) according to the manufacturer's protocol.

2.6. Bisulfite modification and sequencing

Genomic DNA (1 μg) in a volume of 9 μL was denatured with sodium hydroxide (NaOH) at a final concentration of 0.2 M for 15 min, followed by incubation in 200 μL of 2 M sodium bisulfite solution containing 0.24 M NaOH and 10 mM hydroquinone at 50 °C for 4 h. Modified DNA was precipitated with isopropanol and dissolved in 90 μL of water. DNA samples were incubated with NaOH at a final concentration of 0.2 M at 37 °C for 15 min, ethanol-precipitated, and dissolved in TE buffer. To amplify the 319-bp (-134 to +185) hTERT promoter region, bisulfite treated-DNA was subjected to PCR with primer sets (Table 1). PCR amplification was performed using KOD -Multi & Epi- (TOYOBO, Osaka, Japan) for 40 cycles under the following conditions: 98 °C for 10 s, 57 °C for 30 s, and 68 °C for 15 s. After addition of deoxyadenosine to the 3' end by TArget Clone -Plus- (TOYOBO), DNA fragments were cloned into pGEM-T (Promega), and sequenced with T7 and SP6 primers. Tet-assisted bisulfite (TAB) sequencing was performed with the 5hmC TAB-Seq Kit (WiseGene, Chicago, IL, USA) following the manufacturer's instruction. The conversion rate of cytosine to uracil in cytosine residues except for 39 CpG sequences in the 319-bp hTERT promoter region was 97%.

2.7. In vitro DNA methylation and chromatin immunoprecipitation (ChIP) assay

The pGL3-TERT/(+9-+30) × 3 plasmid was methylated by CpG methyltransferase M. SssI (New England Biolabs, Ipswich, MA, USA). DNA methylation was confirmed by digestion with restriction enzymes (BbeI and EcoRI, TaKaRa). Methylated and unmethylated plasmids were introduced into cells by the DEAE-dextran method [17]. ChIP assay was performed as previously described [6,18]. DNA fragments obtained by the ChIP assay were subjected to PCR with primers (Table 1).

2.8. Statistics analysis

A paired *t*-test was performed for statistical analysis. *p* values less than 0.05 were considered significant.

3. Results

3.1. The hTERT promoter is methylated in a human T-cell line, but not normal T-cells

We have previously shown that the transcriptional factor KLF2 represses hTERT gene expression through direct binding to the hTERT

Table 1
Summary of PCR primers.

Usage	Targets	Forward (5'-3')	Reverse (5'-3')
qPCR	hTERT	gaatcagacagcacttgaagagg	cgtagtgagcagcctgaac
RT-PCR	KLF2	gcgccccagcctcggctctc	catgtgcagcgcaggatgat
Bisulfite sequence	hTERT promoter	tagttttgttttttttttttagttt	ccaaccctaaaaaccccaaac
ChIP assay	hTERT promoter	ttcacttcagctccgctcctc	ccccagggcggcagcaaac
ChIP assay	hTERT promoter (+9 to +30) sequences in the pGL3 promoter	caaaatagctgtcccagct	cctcgctcgtcgcataata

promoter in normal resting T-cells [6]. hTERT expression is inversely correlated with KLF2 expression in normal T-cells [6]. To elucidate the molecular mechanism underlying the repression of hTERT expression by KLF2, we measured endogenous and exogenous hTERT promoter activities in the human T-cell line Kit 225 that may regulate cell growth phase by deprivation of IL-2. KLF2 mRNA was expressed in both growing and resting Kit 225 cells, while KLF2 protein level was significantly decreased in growing cells (Fig. 1A). Exogenously transfected-hTERT promoter was significantly activated by IL-2 stimulation, which induced the growth of Kit 225 cells and reduced the protein level of KLF2 (Fig. 1A and B). Although KLF2 protein expression was detected in the resting Kit 225 cells, we failed to notice any down-regulation of endogenous hTERT expression (Fig. 1A and C).

The difference between endogenous and exogenous hTERT promoters prompted us to examine the involvement of epigenetic modification in the regulation of hTERT expression in Kit 225 cells. Previous studies have shown that CpG sequences in the hTERT promoter are hyper-methylated in hTERT-expressing tumor cells and cell lines [13,14,19]. To examine the relationship between hTERT expression and DNA methylation, 5-methylcytosines in the hTERT promoter encompassing the KLF2-binding element were detected by bisulfite DNA sequencing. We analyzed the sequences from -134 to +185 in the hTERT promoter and coding regions, which contained 39 CpG sequences (Fig. 2A and B). The KLF2-binding element includes four CpG sequences (14th to 17th; Fig. 2B). DNA CpG islands in the hTERT promoter showed 70% methylation in growing Kit 225 cells (Fig. 2C). The methylation levels showed no noticeable changes in the absence of IL-2 (Fig. 2C). TAB sequencing exhibited that cytosine residues of the CpG islands in the hTERT promoter were 5-methylcytosines (Fig. 2D). In contrast, the hTERT promoter was unmethylated in normal T-cells examined without any *in vitro* culture (Fig. 2E).

3.2. DNA methylation of the hTERT promoter modulates its gene expression in kit 225 cells

The effects of DNA methylation on hTERT expression were evaluated through the inhibition of DNA methylation with a DNA methyltransferase inhibitor Zebularine, which inhibits the maintenance of cytosine methylation during DNA replication. Zebularine treatment for

6 days in growing Kit 225 cells resulted in the reduction in the methylation of CpG sequences in the hTERT promoter (Fig. 3A). Kit 225 cells deprived of IL-2 following Zebularine treatment significantly reduced the endogenous hTERT mRNA expression (Fig. 3B). The same demethylation treatment had no or minor effect on hTERT expression in growing Kit 225 cells (Fig. 3B). The expression of KLF2 was lower in growing Kit 225 cells than in resting cells irrespective of Zebularine treatment (Fig. 3B). Zebularine treatment had no significant influence on KLF2 expression and cell growth (Fig. 3B and C).

To investigate the involvement of KLF2 in hTERT expression, a siRNA targeting KLF2 was introduced into Zebularine-treated resting Kit 225 cells, and hTERT mRNA levels were measured. Among three species of siRNA used to knockdown KLF2 expression, siKLF2E11 actively repressed KLF2 expression. siRNA-mediated knockdown of KLF2 profoundly increased hTERT expression in demethylation-induced resting cells (Fig. 3D). These results imply that DNA methylation of the hTERT promoter influences hTERT expression in human T-cell line.

3.3. DNA methylation inhibits the binding of KLF2 to the hTERT promoter

To study the effect of DNA methylation on KLF2 association with the hTERT promoter, KLF2 binding to the promoter was examined by ChIP assay. Growing and resting Kit 225 cells were cultured with Zebularine, and the endogenous hTERT promoter was detected by ChIP assay. After demethylation with Zebularine, resting Kit 225 cells clearly exhibited the association of KLF2 to the hTERT promoter, consistent with the reduction in hTERT mRNA levels (Figs. 3B and 4A). No such association was detected with either demethylation-untreated resting and growing Kit 225 cells or demethylation-treated growing Kit 225 cells (Fig. 4A). These results were supported by results of examinations with *in vitro* methylated hTERT reporter plasmid. The pGL3-TERT/(+9-+30) × 3 plasmid carrying only the KLF2 binding element was methylated *in vitro* or remained to be unmethylated. Growing and resting Kit 225 cells were transfected with those plasmids separately and subjected to ChIP assay. KLF2 binding to the unmethylated promoter element in the plasmid was detected in resting cells, but not in growing cells (Fig. 4B). Neither resting nor growing Kit 225 cells exhibited any significant binding of KLF2 to the element in the methylated plasmid (Fig. 4B). These results indicate that DNA methylation obstructs the binding between KLF2 and

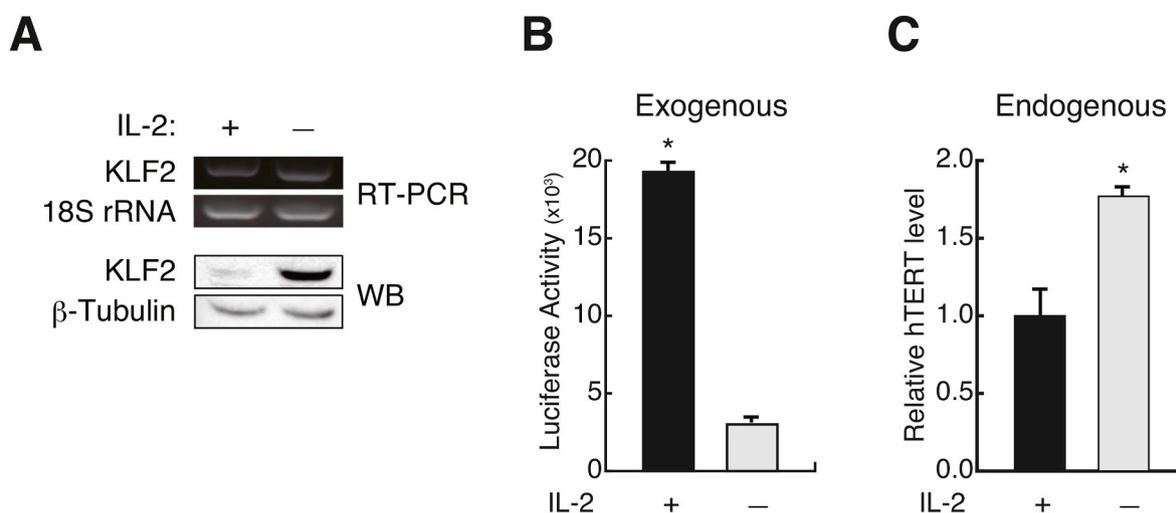


Fig. 1. Differential activity of exogenous and endogenous hTERT promoters in a leukemic cell line. **(A)** Kit 225 resting phase was induced by the cultivation without IL-2 for 2 days. Growing [IL-2(+)] and resting [IL-2(-)] Kit 225 cells were examined for KLF2 and 18 S rRNA gene expression by RT-PCR. KLF2 protein level was monitored by immunoblotting with anti-KLF2 antibody. β-Tubulin was used as an internal control. **(B)** The hTERT promoter reporter plasmid pTERT(-281)-L was transfected into Kit 225 cells. After 40 h of culture with or without IL-2, the cells were harvested for luciferase activity assay and the activity was subsequently normalized against protein content. **(C)** Growing and resting Kit 225 cells were harvested for RNA isolation. The levels of endogenous hTERT mRNA were measured by qPCR. Values are shown as the means of triplicate experiments of the copy numbers ±SE after normalization against 18 S rRNA content. *, $p < 0.05$.

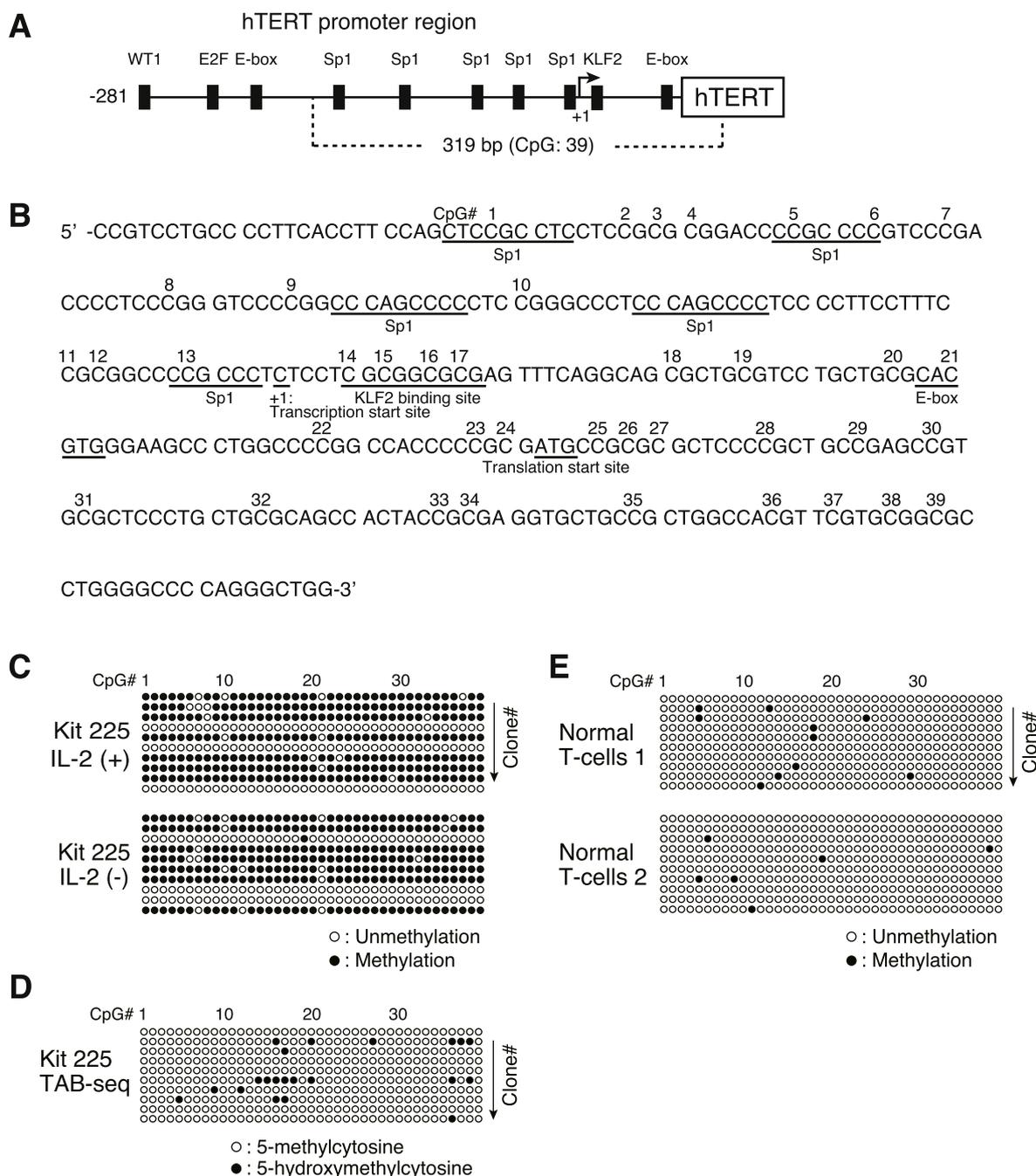


Fig. 2. DNA methylation of the hTERT promoter in Kit 225 cells. **(A)** Schematic representation of the hTERT promoter. Closed boxes indicate transcriptional factor-binding sites that were previously identified [22]. **(B)** Nucleotide sequences of the hTERT promoter. The -134 to +185 region in the hTERT promoter carries 39 CpG sequences. Transcription and translation initiation sites are shown. **(C and E)** DNA methylation of growing and resting Kit 225 cells, and normal T-cells was examined by bisulfite sequencing. Open and closed circles indicate unmethylated and methylated cytosines, respectively. **(D)** 5-Hydroxymethylcytosines of the hTERT promoter were analyzed by TAB-sequencing. Open and closed circles indicate 5-methylcytosine and 5-hydroxymethylcytosine, respectively.

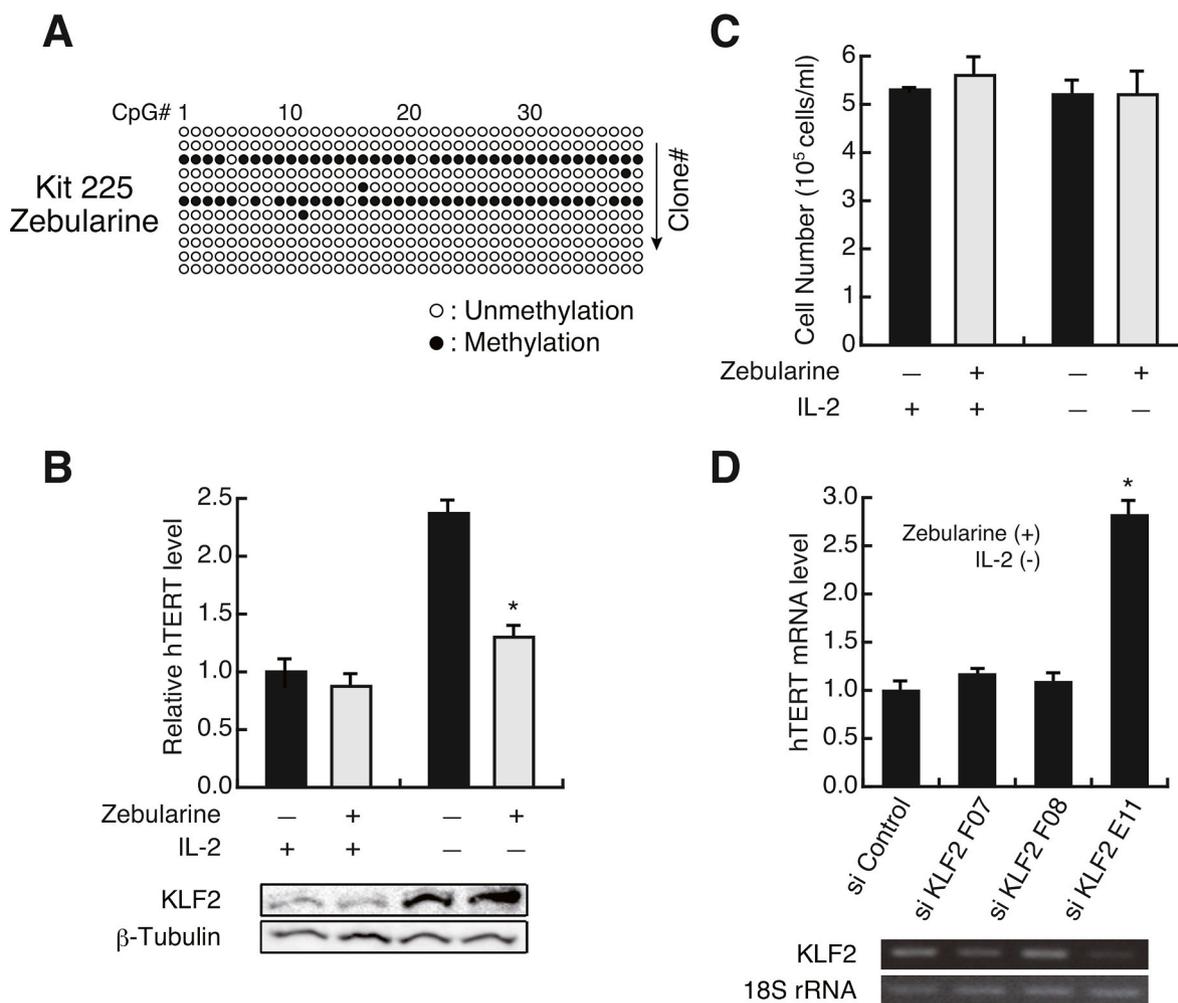


Fig. 3. Effects of DNA methylation on hTERT expression. **(A)** Growing Kit 225 cells were cultured with 10 μM Zebularine for 6 days. DNA methylation was examined by bisulfite sequencing. Open and closed circles indicate unmethylated and methylated cytosines, respectively. **(B–D)** Growing Kit 225 cells were cultured with or without Zebularine for 4 days. Cells were seeded at 1 × 10⁵ cells/mL with IL-2 or at 2.5 × 10⁵ cells/mL without IL-2, and further cultured for 2 days in the presence or absence of Zebularine. Cells were examined for hTERT mRNA expression (B) and cell number (C). Zebularine-treated Kit 225 cells were also subjected to transfection with KLF2-specific siRNA and cultured without IL-2 for 2 days (D). hTERT mRNA expression was analyzed by qPCR (B and D). KLF2 protein level was monitored by immunoblotting with anti-KLF2 antibody. β-Tubulin was used as an internal control (B). KLF2 and 18 S rRNA gene expression was measured by RT-PCR (D). Values are shown as the means of triplicate experiments of the copy numbers ±SE after normalization against 18 S rRNA content. *, *p* < 0.05.

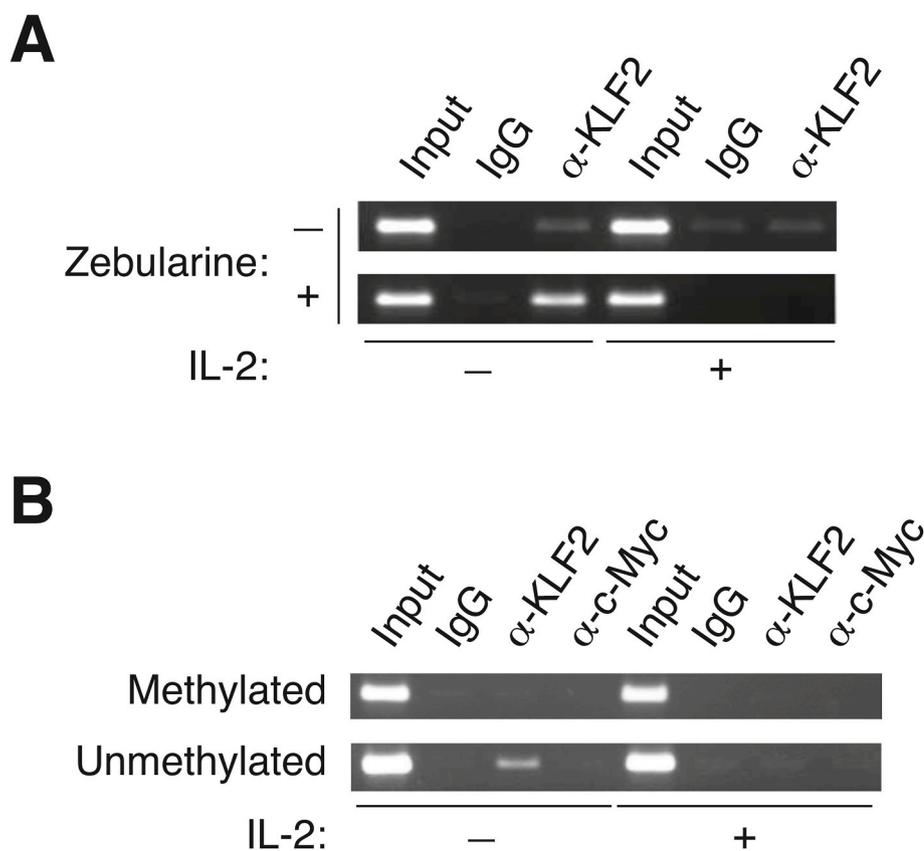


Fig. 4. Binding of KLF2 to the unmethylated hTERT promoter. **(A)** Growing Kit 225 cells were cultured with or without Zebularine for 3 days, and further cultured with or without IL-2 for 2 days in the presence or absence of Zebularine. Cells were treated with or without IL-2 for 1 day in the absence of Zebularine, and subjected to ChIP assays with anti-KLF2 antibody. Experiments were independently repeated five times. Representative results are shown. **(B)** CpG methyltransferase-treated or untreated pGL3-TERT/ (+9-+30) \times 3 plasmid was transfected into Kit 225 cells. Cells were cultured with or without IL-2 for 2 days and subjected to ChIP assays with an antibody against KLF2 or c-Myc.

the hTERT promoter, presumably leading to the transcriptional upregulation of hTERT gene expression.

4. Discussion

DNA methylation of gene promoters in tumor cells has been shown to mostly down-regulate their transcription, as DNA methylation prevents the accession of transcriptional factors to promoter regions [11]. Hypermethylation of promoters of tumor suppressor genes for cell cycle control and mismatch repair molecules usually suppresses their gene expression, leading to tumor progression [20,21]. In this study, we demonstrate that the hTERT promoter is highly methylated in T-cell line that constitutively express the hTERT gene. DNA methylation promotes hTERT gene expression, in contrast to the general understanding of transcription suppression by promoter DNA methylation [11]. Methylation effect is mediated by the inhibition of the binding of the transcriptional repressor KLF2 to the hTERT promoter. The association between KLF2 and the hTERT promoter is recovered by treatment with the DNA methyltransferase inhibitor Zebularine in resting Kit 225 cells along with the reduction in hTERT mRNA expression. These results indicate that the prevention of the association between the hTERT promoter and KLF2 by DNA methylation in T-cell line presumably results in the aberrant expression of the hTERT gene.

The hTERT promoter contains various transcription inducer- and repressor-binding sites for Myc, E2F, Sp1, and CCCTC-binding factor (CTCF) [22]. The E-box at +44 to +49 next to the KLF2-binding element has been reported to bind to the Myc family complexes of Myc/Max and Mad1/Max and mediate transcriptional activation and repression, respectively [23,24]. The Myc family may bind to genomic regions with DNA hyper-methylation [25]. Another regulator involved in hTERT expression, CTCF, binds to the first exon of the hTERT gene, leading to the repression of hTERT expression [26]. DNA methylation at the

CTCF-binding site induces hTERT expression via the inhibition of CTCF binding to the site [27,28]. Thus, the disruption of various hTERT regulation mechanisms in leukemic T-cells leads to the constitutive expression of hTERT.

Our results show that the endogenous hTERT expression in resting Kit 225 cells is higher than in growing cells. The level of cellular mRNA is determined by the balance between mRNA production and degradation. T-cell stimulation results in the rapid decomposition of a large number of mRNAs [29]. KLF2 has no repressive effects on hTERT gene expression in resting Kit 225 cells owing to DNA methylation of its promoter, eventually leading to the accumulation of hTERT mRNA. Therefore, hTERT mRNA may stably exist in resting Kit 225 cells than in growing cells.

Primary cells from childhood patients with acute lymphoblastic leukemia (ALL) were examined for DNA methylation of the hTERT promoter [30]. Methylation of the hTERT promoter at the -201 to -86 region upstream of the KLF2 binding site was seen in 64.7% of T-ALL [30]. This result raises the possibility that hTERT promoter regulation by KLF2 is disrupted in primary T-ALL cells.

In the present study, we clearly demonstrate that DNA methylation inhibits KLF2 binding to the hTERT promoter using Kit 225 cells. Since KLF2 expression oscillates between cell growing and cell resting, the experiment requires a human T-cell line to be arrested at G0/G1 of the cell cycle without appreciable live loss. Only Kit 225 is necessary and sufficient for the purpose among human T-cell lines we tested. Indeed, DNA hyper-methylation of the hTERT promoter is observed in various hTERT-expressing leukemic T-cell lines (unpublished observation). Our findings suggest that DNA methylation of the hTERT promoter induces overexpression of the hTERT gene through the inhibition of KLF2 binding to the promoter in leukemic T-cell lines.

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CRedit authorship contribution statement

Mariko Mizuguchi: Conceptualization, Investigation, Writing – original draft, Funding acquisition. **Toshifumi Hara:** Conceptualization, Investigation. **Manami Yoshita-Takahashi:** Investigation. **Takashi Kohda:** Investigation. **Yuetsu Tanaka:** Investigation. **Masataka Nakamura:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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