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TERT distal promoter GC islands are critical for telomerase and together with DNMT3B silencing may serve as a senescence-inducing agent in gliomas

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ABSTRACT

Telomerase is reactivated in the majority of cancers. For instance, in gliomas, it is common that the TERT promoter is mutated. Research on telomere promoter GC islands have been focused primarily on proximal TERT promoter but little is known about the distal promoter. Therefore, in this study, we investigated the proximal and distal TERT promoter, in terms of DNA methylation. We did bisulfite sequencing in zebrafish tissue samples for the distal tert promoter. In the zebrafish brain tissues, we identified a hypomethylation site in the tert promoter, and found that this hypomethylation was associated with aging and shortened telomeres. Through site directed mutagenesis in glioma cell lines, we changed 10 GC spots individually, cloned into a reporter vector, and measured promoter activity. Finally, we silenced DNMT3B and measured telomerase activity along with vidaza and adriamycin treatments. Site directed mutagenesis of glioma cell lines revealed that each of the 10 GC spots are critical for telomerase activity. Changing GC to AT abolished promoter activity in all spots when transfected into glioma cell lines. Then, through silencing of DNMT3B, we observed a reduction in hTERT expression levels, while hTR remained the same, and a major increase in senescence-associated beta-galactosidase activity. Finally, we propose a model regarding the efficacy of two chemotherapeutic drugs, adriamycin and azacytidine, on gliomas. Here, we show that distal TERT promoter is critical; changing even one GC to AT abolishes TERT promoter activity. DNMT3B, a de novo methyltransferase, together with GC islands in distal TERT promoter plays an important role in regulation of telomerase expression and senescence.

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Telomere; TERT promoter; telomerase; brain cancer; DNA methylation

Introduction

Replicative senescence is the major mechanism behind aging and also the protective mechanism against tumorigenesis. All known DNA polymerases lack the ability to synthesize ends of the linear chromosomes, also known as telomeres, and as a result telomeres get shortened at each cell division. At a critical length, cells stop proliferating and enter a state known as quiescence. Extended periods in quiescence result either in senescence, which is an irreversible state of a non-dividing cell, or bypass senescence and become malignant. The organism has to remain at a delicate balance; it has to prevent malignancy yet to keep senescent cells at a minimum level (Campisi, 2013; Deng, Chan, & Chang, 2008; Sharpless & DePinho, 2004; Zou, Sfeir, Gryaznov, Shay, & Wright, 2004).

Senescent cells are characterized by the absence of proliferation markers, enlarged cell size, presence of a specific enzyme called beta-galactosidase, increased expression of CDKN2A locus genes, as well as tumor suppressors; p53, p21, and PTEN. Not all of these markers are present in a given cell, and cells with a loss of function mutation in

tumor suppressor genes are able to enter senescent state as well (Ozturk, Arslan-Ergul, Bagislar, Senturk, & Yuzugullu, 2009).

Malignant cells usually reactivate telomerase enzyme, to overcome the shortening of telomeres and to bypass senescence. Telomerase is encoded by the gene TERT, which is silenced at the embryonic stages of the mammalian development, in all somatic cells. Telomerase also has an RNA component, encoded by TR gene, yet this gene is kept being expressed at low levels in adult cells. In 85–90% of all tumors, telomerase is reactivated (Kim *et al.*, 1994; Shay, 2016; Wright, Piatyszek, Rainey, Byrd, & Shay, 1996), however activating mutations differ among tumors. In terms of brain cancers, such as primary glioblastoma and oligodendroglioma, TERT promoter mutations account for almost 80% of the TERT mutations, whereas in astrocytoma and neuroblastoma, this ratio is around 10% (Killela *et al.*, 2013).

DNA methylation is a key epigenetic modification involved in gene expression regulation and genome integrity. These heritable and reversible modifications are regulated by DNA methyltransferases (DNMTs). These proteins take the

methyl group from S-adenosyl-L-methionine (SAM) and add it to the C-5 position of cytosines (Robertson, 2005). DNMT1 is known as maintenance DNMT, and preferentially methylates hemi-methylated DNA. DNMT2 is an RNA methyltransferase. DNMT3A and DNMT3B are *de novo* methyltransferases, that is, they add methyl group to CpG sequences that do not contain any methyl group beforehand. DNMT3L, on the other hand, is a protein without enzyme activity, helping DNMT3A and 3B to bind to SAM (Jin & Robertson, 2013).

Methylation is often associated with gene silencing and functions by inhibiting protein binding to the target DNA sequence. However, the case is not that simple for TERT promoters. Besides the conflicting results, many of the studies agree that there are two different regions in TERT promoter responsible for gene regulation: upstream of the transcription start site (UTSS) (proximal) and TERT hypermethylated oncological region (THOR) (distal) (Lee, Komosa, Nunes, & Tabori, 2020). While the distal THOR region (572–140 bp to TSS) is methylated, the core (140 bp upstream of TSS) region remains unmethylated in many cancer cells such as prostate, bladder, thyroid, and lungs (Lee *et al.*, 2019).

There are some speculations about this behavior of the TERT promoter. One of them is that this methylation can prevent repressors from binding to the promoter. Another estimation is chromomeric changes caused by CTCF protein may lead a distal enhancer to interact with the promoter (Lee *et al.*, 2020). Within these results, it is possible to say that epigenetic regulation of the TERT promoter has a remarkable role in malignant formation and/or maintenance. In the review by Dogan *et al.*, it is possible to read more about the telomerase regulation through epigenetics (Dogan & Forsyth, 2021).

In this study, we observed a hypomethylation site in the tert promoter of zebrafish brain, which has not been reported before, and found that this was associated with shortened telomeres and aging. We confirmed this finding with an additional cohort of zebrafish brain samples. The potential role of epigenetic changes on human TERT promoter in cancer was reported before in many studies (de Wilde *et al.*, 2010; Iliopoulos, Oikonomou, Messinis, & Tsezou, 2009; Kumari, Srinivasan, Vasishtha, & Wig, 2009). However, in brain samples, only UTSS-proximal region was studied (Castelo-Branco *et al.*, 2013). Here, we studied greater region of the promoter which also includes both proximal and distal parts, and through site-directed mutagenesis, we identified hotspots that abrogate promoter activity in two glioma cell lines. Then through silencing of DNMT3B, we propose a senescence-inducing, therapeutical option, which can replace azacytidine and adriamycin treatments.

Materials and methods

Methods for zebrafish experiments

Tissue collection

Wild-type zebrafish (AB) was used for tissue collection. All fish were kept in Techniplast aquarium system, with a

14:10 h light:dark cycle. All fish were fed *ad libitum*, twice with dry flakes and once with Artemia, on a daily basis. Detailed information on all the fish used in this study is given in [Supplementary Tables S1 and S2](#). Experiments are performed according to the rules determined by Bilkent University Animal Ethics Committee and approved with approval number of 2014/18.

Bisulfite conversion

EpiTect Fast DNA Bisulfite kit (Qiagen) and EZ DNA methylation kit (Zymo Research) were used according to manufacturer's instructions. Zebrafish TERT promoter sites are amplified following bisulfite treatment for sequencing. ZymoTaq Premix (Zymo Research) is used for PCRs. Tert-CpG F: 5'-ATA GTA GGA TAG GGT TTT GGT TTT G-3', and Tert-CpG R: 5'-CCT TCA ATT CTT CAA AAA ATT AAC TC-3' are used for promoter amplifications. Amplified reactions are sent for Sanger sequencing. Sequence alignments are done on ApE-A Plasmid Editor v2.0 (by Wayne Davis) to reference sequence obtained from ZFIN Data on GRCz10 for TERT gene.

Methods for human cells

Cell culture

Cell lines were cultured according to suggestions from ATCC's website. All cell lines are cultured in DMEM medium (BioWest) with 1% penicillin–streptomycin (Gibco), 1% L-glutamine (Gibco), 1% nonessential amino acids (BioWest), and 10% FBS (BioWest). Cells were routinely checked for mycoplasma contamination using BioWest's MycoKit.

PCR for TERT promoter sites

PCRs were performed for amplifying core and distal TERT promoter area from different cell lines while adding restriction enzyme recognition site at each end of the product. Thermo Scientific's Phusion High Fidelity Master Mix with High GC content is used for reactions. To increase restriction enzyme digestion efficiency, six random nucleotides are added before the restriction enzyme recognition site by PCR. KpnI-HF (NEB), and SacI-HF (NEB) are used for pGL3-Basic (Promega), and TERT promoter digestion. DpnI-HF (NEB) is used for digestion of template DNA from site-directed mutagenesis PCRs.

Site directed mutagenesis of TERT promoter

Used primers, and final concentration used in PCRs are shown in [Supplementary table: SDM primers](#). 2x Phusion Mater Mix with High GC (Thermo Scientific) is used for mutagenesis PCRs.

PCRs were performed for amplifying core, and distal TERT promoter area from different cell lines while adding restriction enzyme recognition site at each end of the product. To increase restriction enzyme digestion efficiency, six random nucleotides are added before the restriction enzyme

recognition site by PCR. Primer pair for core promoter was TERT 4F: 5'-GTA ATA GGT ACC TCG CCG CAC GCA-3', and TERT 2R: 5'-ATG TAG GAG CTC CAC GAA CGT GGC-3'. For distal promoter area, TERT 5F: 5'-GTA TGA GGT ACC ACC AGT GGC CGT G-3' with TERT 2R. Thermo Scientific's Phusion High Fidelity Master Mix with High GC content (F-532, Thermo Scientific) is used for reactions. PureLink PCR purification kit (K310001, Invitrogen) is used for PCR products. pGL3-Basic Luciferase Reporter Vector (E1751, Promega) was purchased. KpnI-HF and SacI-HF restriction enzymes were used for pGL3-Basic and TERT promoter digestion. For each digestion reaction, 1 µg of DNA is used. Zymo DNA clean and concentrator kit (D4034, Zymo Research) was used according to manufacturer's protocol with minor modifications to clean up restriction enzyme digested pGL3-Basic, and TERT samples prior to ligation reaction. 1:3 molar ratio of plasmid: insert is used in core promoter ligation reactions, and 1:5 molar ratio is used for distal ligation reactions. All calculations were made using NEBioCalculator (New England Biolabs). Length of pGL3-Basic is 4818 bp. Insert length is 538 for new TERT 2R-4F reaction (core promoter), and 1150 for new TERT 2R-5F reaction (distal promoter). Ligation and DpnI-HF digestion products were directly used for transformation without further isolation steps. Chemically competent Jm109 strain of *Escherichia coli* was used for transformation.

Transfection and luciferase assay

Per each well of a 6-well plate, 200 µl of serum free DMEM F12 is mixed with 2 µg plasmid DNA, and 4 µl transfection reagent (ATCC). After 24 h, cell culture lysis reagent (Promega) is added, culture plates are rocked several times, and then the cells are scraped. A 20 µl cell lysate is added to luminometer tube, and mixed by pipetting up, and down for 3 times. The tube is placed in the luminometer, read is initiated, and recorded.

siRNA transfection

DNMT3B and Non-Targeting ON-TARGETplus siRNAs are purchased from Dharmacon, GE Healthcare. Cells are incubated 48 h post transfection for gene expression analysis and 96 h post transfection for protein analysis.

Azacytidine and adriamycin treatment of cell lines

Azacytidine (Er-kim) and Adriamycin (Saba İlaç) treatments are used as positive controls for beta-galactosidase staining experiments. Mediums with anticancer drug are replaced with complete medium at the 24th hour and cultured until 96 h post treatment.

Quantitative polymerase chain reaction

At 48-h post siRNA transfection, RNeasy Mini Kit (Qiagen) is used for RNA isolation from cell lines, and all steps are

performed at room temperature. Transcriptor HighFidelity cDNA synthesis kit (Roche) is used for cDNA synthesis. Sensifast SYBR No ROX 2x master mix (Bioline) is used for qPCR experiments. Primers are listed in [Supplementary Table S3](#). Gene expression is calculated by normalizing to RPL13A (neuro-housekeeping gene) and $\Delta\Delta C_t$ method.

Beta galactosidase staining

SA β -gal staining kit (9860S, Cell Signaling Technologies) is used according to manufacturer's suggestions. Cells are imaged using Olympus DP71 CKX41. Image quantification is done using Fiji (ImageJ) software.

Telomere length measurement

Fast Start SYBR Green Master (Roche) is used for telomere length measurements in zebrafish tissues according to the protocol described earlier (Arslan-Ergul, Erbab, Karoglu, Halim, & Adams, 2016). Primer pairs are listed in [Supplementary Table S3](#). Relative telomere length is calculated by the formula $2^{-(C_{telo}-C_{t36b4})}$.

Quantitative telomeric repeat amplification protocol

For each assay, 300,000 cells were used. Freshly prepared 1x Chaps Lysis is added. Samples are incubated for 30 min on ice, and then centrifuged at 14,000 rpm for 20 min at 4 °C. Cell lysate is transferred into a new tube, aliquoted, and stored at -80 °C until use. ACX: 5'-GCG CGG CTT ACC CTT ACC CTT ACC CTA ACC-3' and TS: 5'-AAT CCG TCG AGC AGA GTT-3' used as primers. Calculations are made by plotting a standard curve using positive control dilution series vs. log₁₀ of number of cells.

Statistical analysis

The GraphPad Prism 8 (GraphPad Software, Inc.) was used for statistical analysis. For zebrafish experiments, the homogeneity of variance is tested with Kolmogorov-Smirnov and Shapiro-Wilk tests. For cases with equal variance, univariate analysis of ANOVA is used, followed by Tukey's test. For cases with unequal variance, nonparametric Kruskal-Wallis test is used, followed by Dunn's test. For human cell line experiments, homogeneity of variance is tested with Shapiro-Wilk tests. In Luciferase assay experiments, RM one-way ANOVA with Geisser-Greenhouse correction and Sidak's multiple comparison is used for data analysis. In population doubling time experiments and beta-galactosidase staining experiments, ordinary one-way ANOVA with Dunnett's multiple comparisons is used for data analysis. In quantitative telomeric repeat amplification protocol (qTRAP) experiments, ordinary one-way ANOVA with Geisser-Greenhouse correction and Sidak's multiple comparisons is used for data analysis. In gene expression analysis experiments, ordinary one-way ANOVA with Dunnett's multiple comparisons is used in the analysis. In all experiments, a cutoff of $p < 0.05$ is used for statistical significance determination.

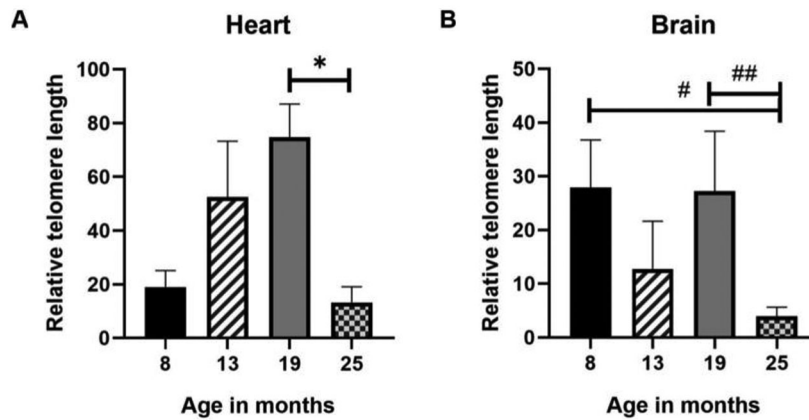


Figure 1. Relative telomere length in aging zebrafish (A) heart and (B) brain. X-axis shows the age in months and Y-axis shows the relative telomere length. For brain, n (8 months)= 6, n (13 months)= 5, n (19 months)= 5, n (25 months)= 6; for heart, n (8 months)= 7, n (13 months)= 5, n (19 months)= 4, n (25 months)= 5. Error bars represent the + SEM. *: $p = 0.03$, #: $p = 0.0113$, and ##: $p = 0.0241$.

Results

Telomere lengths shorten in zebrafish brain and heart at a certain age

We first compared the telomere lengths in different organs of aging zebrafish. In heart and brain, we found significant differences between 19 and 25 months of age. We observed that the telomere length dropped drastically (~83–85%) somewhere between 19 to 25 months of age. Additionally, in the brain there was a significant difference between 8 and 25 months (Figure 1).

For the other organs, we did not observe any significant change (Supplementary Figure S1).

In order to understand the high variation between individuals, and the drastic change in the telomere length in 25 months of age, we performed bisulfite sequencing for brain, skin, muscle, and spleen samples. Since we used individual samples, other tissue samples were not fitted for bisulfite reaction due to small sample sizes. We analyzed the distal tert promoter region and found a specific hypo-methylation site in the brains of old animals at chromosome 19 position 627,118 (–962). Two of the four old samples had the hypomethylation, whereas none of the young. In order to test our findings in another cohort, we used 16 zebrafish brain DNA samples from our earlier work (details of the fish are given in Supplementary Table S2) (Arslan-Ergul *et al.*, 2016).

Figure 2 summarizes the methylation status and relative telomere lengths of a total of 9 old and 17 young zebrafish brain samples. In Supplementary Data S1, sequence alignment of all samples is given as aligned to the reference sequence, which is the PCR product without bisulfite conversion. The fish used in this analysis and the methylation status are mentioned in Supplementary Tables S1 and S2. Alignment indicated eight CpG sites available for methylation (highlighted in the alignment file).

All young fish exhibited 100% methylation in this loci, whereas the methylation in 7 of the 9 older fish ranged from 62.5% to 87.5%, with the –962 and –981 region upstream of the TSS exhibiting hypomethylation in 6 of the 9 older fish, indicating that hypomethylation of this region occurs as

a result of age (details of the calculations are given in Supplementary Data S1).

On the other hand, none of the young samples had the hypo-methylation; all had a methyl attached to the CpG site in all the positions. In terms of telomere lengths, individual variation aside, old samples had overall lower telomere lengths. Thus, our initial findings point that there is a specific methylation site, which loses its methylation during aging, and is related to shorter telomere lengths. Also, this event is exclusive to the brain tissue.

Human brain cancer cell lines secure methylation in specific sites throughout the TERT promoter

The position where we observed hypomethylation in zebrafish brain tissues is a predicted Sp1 transcription factor binding site through in silico analysis (Broos *et al.*, 2011). We run the sequence that we amplified after bisulfite treatment and found the possible Sp1 binding sites with Contra

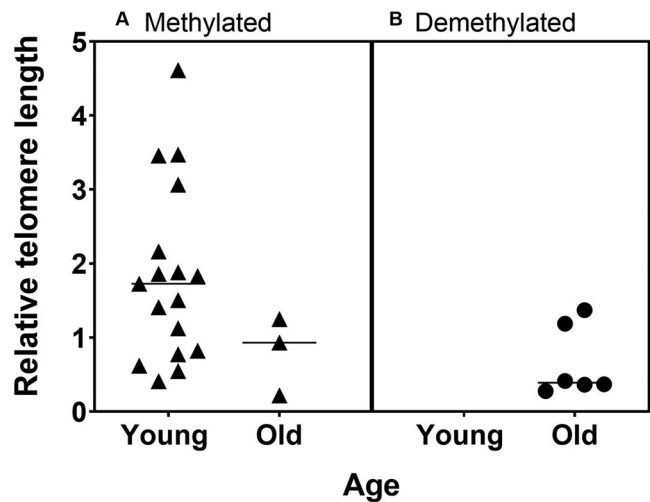


Figure 2. Relative telomere length and methylation status in –962th position of TERT promoter in young (8–8.5 months) and old (26–32.5 months) zebrafish brains. Y-axis shows the relative telomere length and X-axis shows the age groups. Individual data points are shape coded according to their methylation status, where methylation is shown with triangles and hypomethylation is shown with circles. n (young)= 17, n (old)= 9. Error bars represent the + SEM.

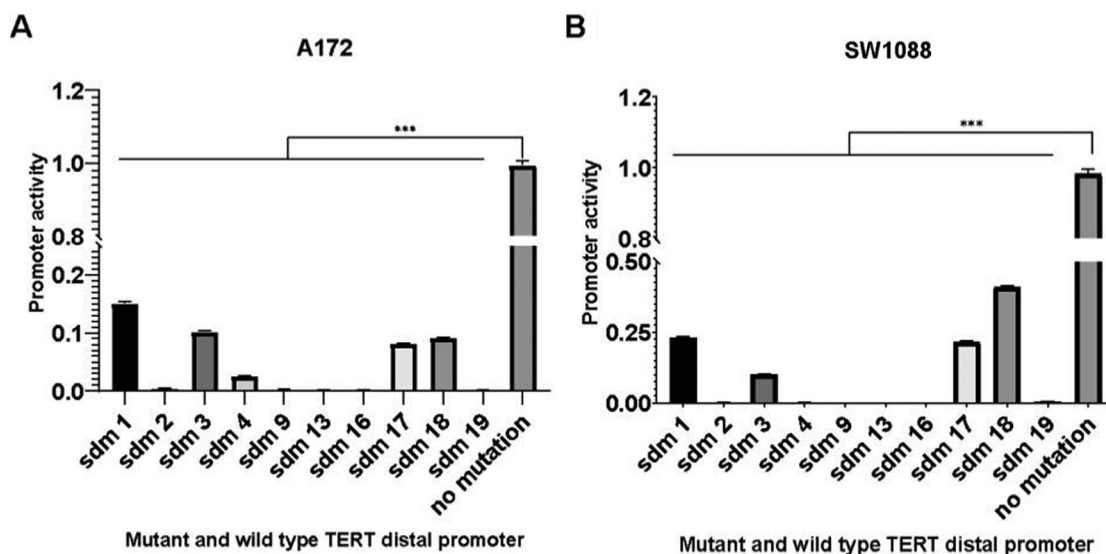


Figure 3. TERT promoter activity after site-directed mutagenesis in brain cell lines. Y-axis shows the relative promoter activity and X-axis lists the mutant and wild type TERT distal promoter. (A) A172. (B) SW1088. No mutation value is set to 1. $n = 3$ for all measurements. Error bars represent the \pm SEM. ***: $p < 0.001$.

v2 tool. The original sequence, which we obtained from NC_007130.7 has Sp1 binding site predicted at this spot, however, bisulfite-treated version did not. So, we hypothesized that the hypomethylation changes the binding ability of Sp1, which may result in reduced expression of telomerase. In order to test this hypothesis, we utilized human brain cancer cell lines and performed site-directed mutagenesis (SDM). We inserted mutations to the predicted-Sp1 binding sites in human TERT promoter and cloned these into pGL3 plasmid vectors. Then, we transfected brain cancer cell lines, A172 (glioblastoma) and SW1088 (astrocytoma) with these constructs and measured the luciferase activity after 24 h. Effects of the mutations on the promoter activity are given for A172 and SW1088 (Figure 3 and Table SDM). SDM 2, 9, 13, 16, and 19 completely abrogated the promoter activity in all cell lines. SDM 3 and 4, reduced the activity to a great extent with varying levels (10-fold to full inhibition), and SDM 1, 17, and 18 reduced the activity at moderate levels (2-fold to 20-fold) in different cell lines (Figure 3). All changes were statistically significant when compared with no-mutation control. These data clearly showed that the methylation sites in the TERT promoter are critical, and that preventing the methylation site abrogates the promoter activity.

Inhibition of de novo methyltransferase DNMT3B decreases hTERT levels while leaving hTR unchanged

The SDM sites were predicted to be the Sp1 binding sites, and it is possible that preventing the binding of Sp1 leads to promoter activity blockage. We aimed to analyze more Sp1 binding sites and also to perform bisulfite sequencing, however, we were unable to perform these tasks due to high CpG content of the region. In order to analyze the effect of methylation on TERT expression, we decided to block methylation globally; via DNMT3B siRNA and azacytidine treatment. Azacytidine (vidaza) is a hypomethylation agent,

which prevents all methylation in a cell. DNMT3B, as being the de novo methyltransferase, enables addition of methyl residues. First, we wanted to compare the basal TERT expression levels in our cell lines. VA-13 cell line has been shown before to be telomerase deficient (Cerone, Autexier, Londoño-Vallejo, & Bacchetti, 2005). In our data also it had almost undetectable levels of TERT, which was as expected and SW1088 had lower levels than A172 (Figure 4(A)). Although not statistically significant, azacytidine lowers TERT expression in A172, yet increases in SW1088. When DNMT3B is silenced, TERT gene expression is reduced dramatically in both cell lines (Figure 4(B,C)). In contrast to their effect on TERT, azacytidine lowers hTR expression in all cell lines, including VA-13, whereas DNMT3B had no effect (Figure 4(D-F)). Thus, although azacytidine has differential effect on cell lines, DNMT3B silencing is consistent. Also, DNMT3B silencing acts only on TERT levels and not on TR levels.

DNMT3B silencing drives brain cancer cells into senescence

To investigate the effect of DNMT3B inhibition on telomerase, we performed qTRAP experiment. HEK293T cells were used with a serial dilution as the positive control and VA-13 as the negative control. A172 and SW1088 cells had very low levels of telomerase activity, when compared with the HEK293T cells (Figure 5(A)). Still, DNMT3B silencing significantly reduced the telomerase activity in comparison to the scrambled control group in both cell lines (Figure 5(B)).

Adriamycin and azacytidine are known inducers of senescence in cancer cells (Elmore *et al.*, 2002; Sung, Lee, Kim, & Choi, 2018; Venturelli *et al.*, 2013; Weller, Poot, & Hoehn, 1993). Here we tested these two drugs on A172 and SW1088 cells, along with DNMT3B silencing. Two cells responded to the drugs differentially. Adriamycin induced senescence in A172 cells, but not in SW1088, whereas azacytidine acted opposite. It induced senescence in SW1088 cells, but not in

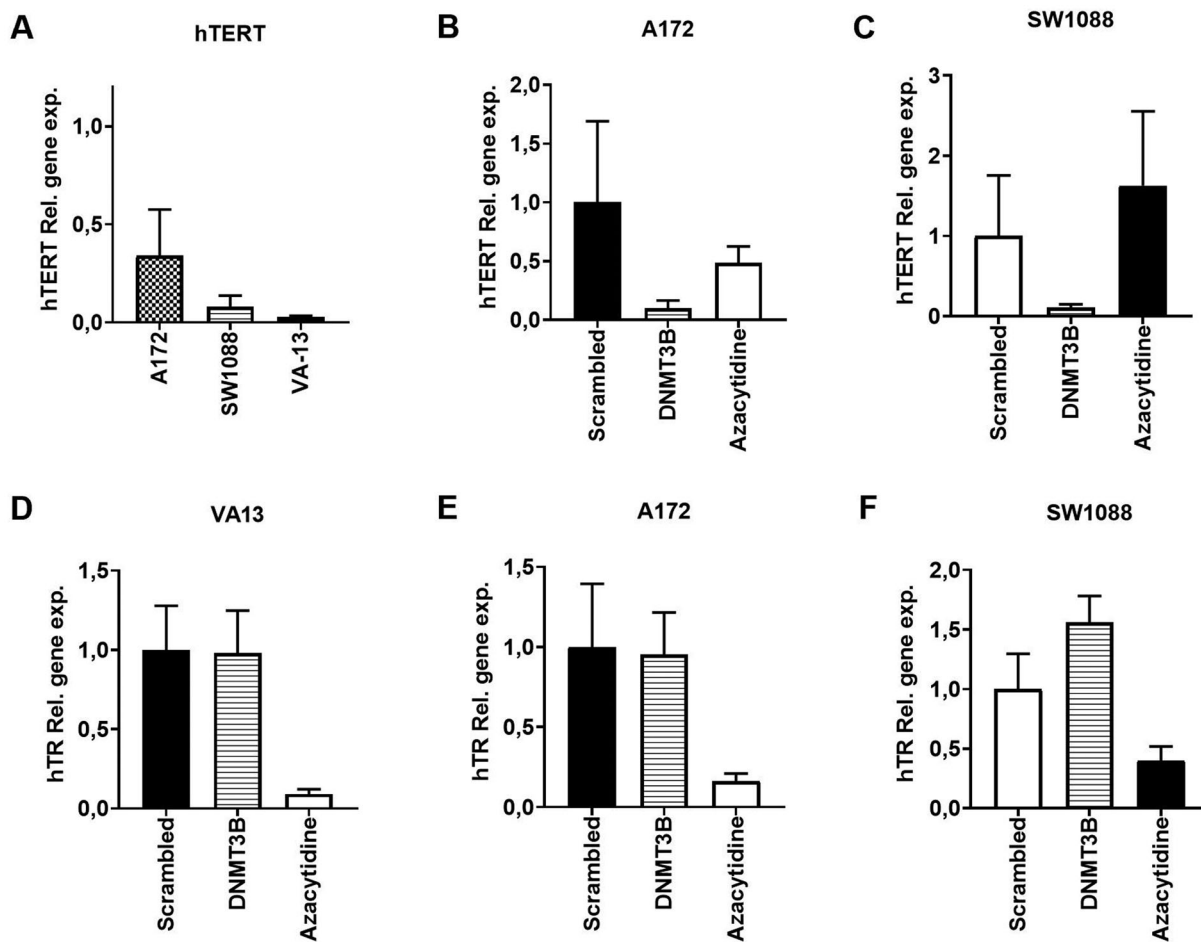


Figure 4. Expression of hTERT and hTR in brain cancer and control cell lines. Y-axis shows the normalized relative gene expression and X-axis shows the treatment type. Final concentration of the treatments are 25 nM for siRNAs and 10 μ M for Azacytidine. (A) hTERT expression in comparison between cell lines before treatments and (B) A172, (C) SW1088 after DNMT3B siRNA and azacytidine treatment. hTR expression in (D) VA-13, (E) A172, and (F) SW1088 after DNMT3B siRNA and azacytidine treatment. Scrambled is set to 1. $n = 4$ for all measurements. Error bars represent the + SEM.

A172 cells. DNMT3B silencing on the other hand, induced senescence in both cell lines (Figure 5(C,D)). Population doubling times were not affected by DNMT3B silencing (Supplementary Figure S3). Thus, although two anticancer drugs act differentially on two different cell types, DNMT3B silencing can serve as a common therapeutic option for two different tumors.

Discussion/conclusion

We had presented in our previous work (Arslan-Ergul *et al.*, 2016; Erbaba *et al.*, 2020) that (1) telomere lengths shorten in all organs throughout aging, (2) there is a variation among individuals, and (3) among organs of the same animals, and we verified these findings here. In the current study, and to our knowledge for the first time, we found that a specific CpG location in zebrafish tert promoter (-962 from the transcription start site) has a role in aging; in aged brains this site is hypomethylated.

As a next step, one of the proteins that may have the potential to enact epigenetic changes on TERT promoter was studied: DNMT3B. We found that when DNMT3B is silenced, brain cancer cells enter senescence. Moreover, there is a significant decrease in hTERT expression level. This finding is consistent with the *in vivo* result of Huang and his colleagues. In 2017, they found that knocking-down of DNMT3B causes stalling of growth in pancreatic cancer (Wang *et al.*, 2018). Another study (Ciccarone *et al.*, 2016) showing the relationship of DNMT3B and aging demonstrated that this protein's expression level decreases with the increasing age especially at females.

In addition to the possibility that DNMT3B decrease can cause the hypomethylation of TERT promoter, it may be the result of the decreased TERT protein level. This dual activation of TERT and DNMT3B was revealed by Yu *et al.* in 2019 (Yuan & Xu, 2019). They hypothesized that there is a positive feedback loop between these two protein. While the DNMT3B expression is promoted by the cooperation of TERT and Sp1, DNMT3B can cause the activation of TERT, which we add another proof. Actually this positive feedback

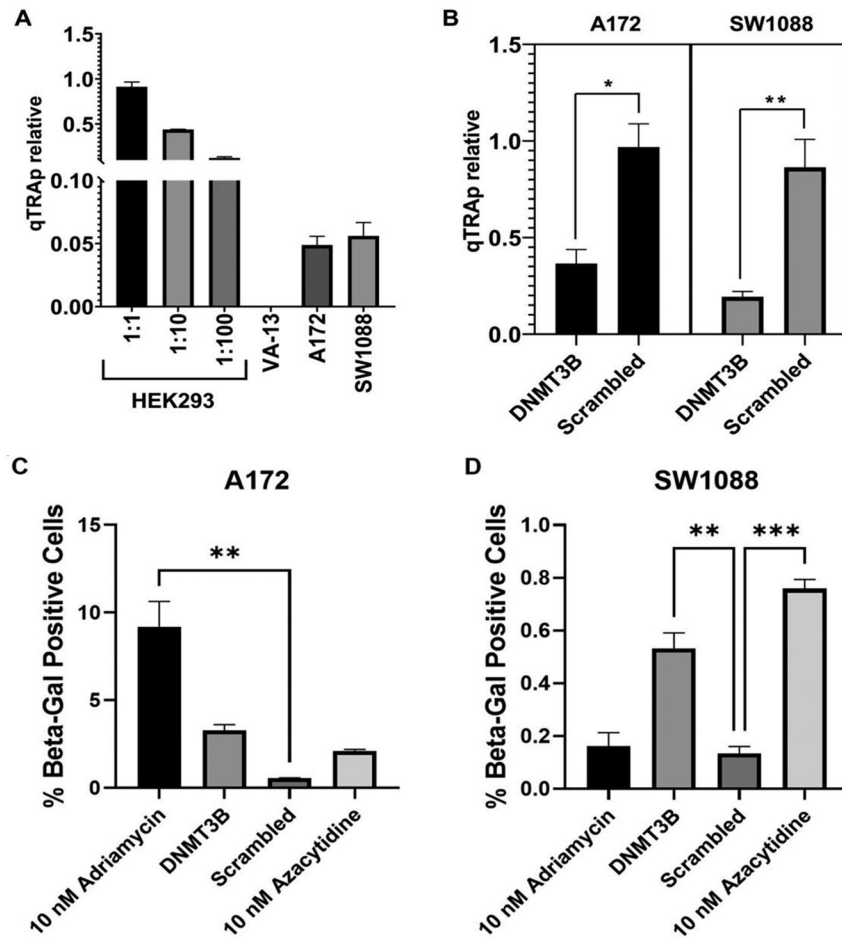


Figure 5. Telomerase activity and senescence assay in DNMT3B-silenced cells. (A) Comparative telomerase activity levels in different cell lines. Y-axis show the relative telomerase activity and X-axis shows the cell type and treatment. HEK293T is used for positive control. Serial dilution of HEK293T is required for standard curve generation, where linear equation is used for relative telomerase activity measurement. (B) Effect of DNMT3B-silencing treatment on telomerase activity. as *: $p = 0.0195$ and **: $p = 0.0391$. Beta-galactosidase staining in (C) A172 and (D) SW1088 brain cancer cell lines. Y-axis shows the percentage of beta-galactosidase positive cells and X-axis shows the treatment. Final concentration of 25 nM siRNA is used for transfection. Scrambled is nontargeting siRNA. Error bars represent the \pm SEM. $n = 3$ for all measurements. Error bars represent the \pm SEM. *: $p < 0.05$, **: $p < 0.001$, and ***: $p < 0.0001$.

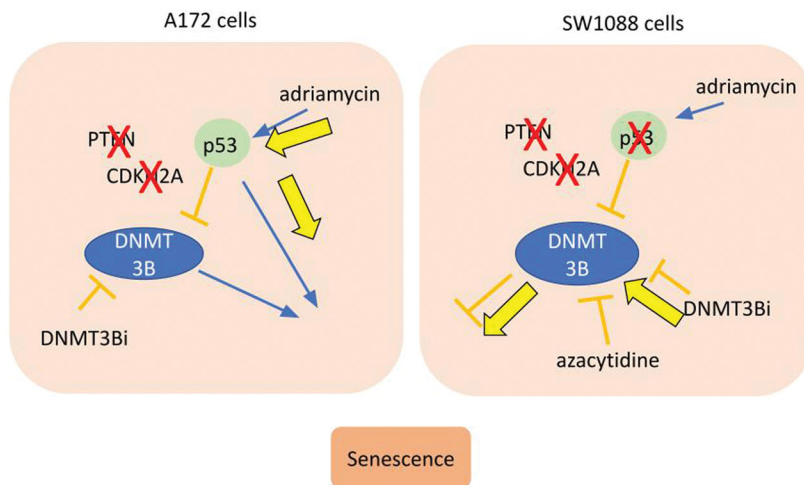


Figure 6. DNMT3B inhibition induces senescence in p53 deficient cells. In A172 cells (left), PTEN and CDKN2A is lost, but p53 remains intact. DNMT3B is suppressed by p53, which relieves DNMT3B suppression on senescence. Adriamycin acts through p53 and induces senescence (yellow arrows). DNMT3B inhibition and azacytidine also induces senescence, but not as strongly as adriamycin. In SW1088 cells, p53 is missing and adriamycin cannot induce senescence in these cells. DNMT3B is no longer suppressed by p53 can now be a target of azacytidine and DNMT3B inhibitors. DNMT3B's inhibition on senescence pathways is released upon DNMT3B silencing. Hence DNMT3B inhibition serves as a common inducer of senescence in both cell lines.

loop can also account for why we see greater reduction in hTERT level in the case of DNMT3B silencing rather than in comparison to azacytidine treatment which is a global hypomethylator.

The −962 region is an Sp1 binding site (through in silico analysis) and we found that it is hypomethylated in aged zebrafish brain. Sp1 is a transcription factor, which regulates tert expression both directly and indirectly through c-myc and NF-κB. We hypothesized that Sp1 binding is critical for telomerase expression, even at a distal site, and that through hypo-methylation, a novel senescence inducing component is found. We wanted to test our hypothesis on human brain cancers because human brain cancers, especially gliomas are enriched in TERT promoter mutations and have over 90% rate of telomerase re-activation. Two cell lines that we utilized, A172 and SW1088 cells are both glioma-derived cells and lack CDKN2A and PTEN expression. SW1088, in contrast to A172, lack p53 expression and is tumorigenic in nude mice. In these two cell lines, through site-directed mutagenesis, we found that 10 CpG islands, are critical for TERT promoter activity. We targeted 20 CpG sequences from translation start site to the 1 K upstream. Of these 20, we were able to introduce mutations in 10, and unable to achieve remaining sites due to high CpG content of the region. Of the 10 mutations, all had significantly abrogated the promoter activity. All mutations were designed to prevent the methylation at the target site. As we gather from this finding, not only the proximal region, but also the distal region of the tert promoter is critical for gene activity and this is mediated through methylations and possibly through Sp1 binding.

Adriamycin induces senescence through p53. In our senescence assay, we observed that it induces senescence in A172, but not in SW1088, which is p53 negative. Azacytidine on the other hand, is expected to block methyltransferases and telomerase. A172 has higher telomerase activity than SW1088 (Figure 5(A)), which may explain why azacytidine do not induce senescence. Azacytidine induces senescence via p16 upregulation, yet SW1088 is CDKN2A deficient, so the mechanism should be through a different pathway (Figure 6).

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Ethical approval

Experiments are performed according to the rules determined by Bilkent University Animal Ethics Committee and approved with approval number of 2014/18.

Author contributions

Naz Serifoglu contributed to conceptualization, methodology, and writing—original draft. Begun Erbaba contributed to methodology and investigation. Michelle Adams contributed to investigation and

writing—review and editing. Ayca Arslan-Ergul contributed to conceptualization, writing—original draft, and funding acquisition.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

Data are available in Mendeley: Arslan Ergül, Ayça (2021), “DNMT inhibition”, Mendeley Data, V1, doi: 10.17632/n387s8kpt5.1 and Arslan Ergül, Ayça (2022), “Serifoglu J neurogenet”, Mendeley Data, V1, doi: 10.17632/zvpzvjjzfr.1.

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