The effect of chemotherapeutic agents on telomere length maintenance in breast cancer cell lines

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Abstract Mammalian telomeric DNA consists of tandem repeats of the sequence TTAGGG associated with a specialized set of proteins, known collectively as Shelterin. These telosomal proteins protect the ends of chromosomes against end-to-end fusion and degradation. Short telomeres in breast cancer cells confer telomere dysfunction and this can be related to Shelterin proteins and their level of expression in breast cancer cell lines. This study investigates whether expression of Shelterin and Shelterin-associated proteins are altered, and influence the protection and maintenance of telomeres, in breast cancer cells. 5-aza-2'-deoxycytidine (5-aza-CdR) and trichostatin A (TSA) were used in an attempt to reactivate the expression of silenced genes. Our studies have shown that Shelterin and Shelterin-associated genes were down-regulated in breast cancer cell lines; this may be due to epigenetic modification of DNA as the promoter region of POT1 was found to be partially methylated. Shelterin genes expression was up-regulated upon treatment of 21NT breast cancer cells with 5-aza-CdR and TSA. The telomere length of treated 21NT cells was measured by q-PCR showed an increase in telomere length at different time points. Our studies have shown that down-regulation of Shelterin genes is partially due to methylation in some epithelial breast cancer cell lines. Removal of epigenetic silencing results in up-regulation of Shelterin and Shelterin-associated genes which can then lead to telomere length elongation and stability.

Keywords Shelterin · Telomere · 5-aza-CdR · TSA · Telomerase

Introduction

Telomeres are made up of G-rich nucleotide repeats of the sequence (TTAGGG)_n bound by associated proteins at the ends of the chromosomes of eukaryotic and all mammalian cells [1, 2]. Telomeric DNA together with the associated telosomal proteins, collectively known as the Shelterin complex, is essential for the overall maintenance of genome integrity and prevents DNA degradation and chromosome end-to-end fusions [3]. The Shelterin or telosome, consist of the six proteins TRF1, TRF2, POT1, TIN2, TPP1 and RAP1, it packages telomeric DNA and helps to hide the chromosome ends from being recognized as sites of DNA damage during DNA replication [4]. TRF1 and TRF2 bind to the double stranded T-loop of telomeric DNA and are implicated in maintaining the formation of the T-loop structure, while POT1 interacts with single-stranded TTAGGG repeats at the 3’ overhang, as well as in the D-loop of the T-loop configuration. TRF1 and TRF2 recruit TIN2, RAP1, TPP1, and POT1 to telomeric DNA [4]. Shelterin proteins interact with a number of other factors known as Shelterin-associated proteins that can influence chromosome end integrity and dynamics. These Shelterin-associated proteins are known as TNKS1, TNKS2, SMG6, and TEP1 [5–7]. Telomerase is a unique cellular ribonucleoprotein (RNP) complex that synthesizes TTAGGG sequence repeats onto the 3’ end of chromosome terminals [1, 8]. The core enzyme contains two subunits: telomerase reverse transcriptase.
(TERT) and telomerase RNA (TR) [9]. The TR molecule, complementary to the telomeric repeats, is an important element of the telomerase enzyme; it consists of an RNA template region that facilitates adding telomeric repeats through the action of the reverse transcriptase catalytic subunit of telomerase (TERT) [10]. These two subunits can bind with additional proteins that together expedite synthesis and elongation of telomeric DNA [8]. In the absence of telomerase, i.e., in most normal adult somatic cells, the hexanucleotide repeats decrease after each cell division; therefore cells undergo senescence when the lengths of telomeres are reduced to a critical level. In addition, telomere loss causes genome instability, resulting in destruction of cell-cycle control, one of the hallmarks of cancer [11]. In most cancer cells, telomerase has been reactivated and prevents cancer cells from entering senescence [11]. Consequently, the activation of telomerase is an important step in development of human cancers [7]. In addition, telomere dysfunction through telomere shortening and/or dysregulation of telomeric DNA-binding proteins (Shelterin), occurs in both the in situ and invasive stages of many cancers, such as breast cancer [12]. Previous noteworthy studies reported different expression levels of Shelterin and Shelterin-associated genes in different human cancers [7, 11, 13]. Salhab et al. [7] indicated over-expression of TNKS1, hTERT, EST1, and TEP1 and down-regulation of TNKS2 and POT1 mRNA levels in breast cancer tissues compared with normal breast tissues. Moreover, the lower expression of TRF1 and TRF2 was found to be associated with the development and progression of breast cancer. However, findings by Hu et al. [14] in other cancers appeared to be contradictory to this. They demonstrated significant over-expression of TRF1, TRF2, and TIN2 in precancerous lesions, gastric cancer tissues, and lymph node metastases in comparison with normal gastric mucosa tissues [14]. In addition, recently published data by Lu et al. [11] showed over-expression of POT1 mRNA levels in gastric cancer tissues.

Epigenetic modifications such as abnormal DNA methylation and modifications of histones play an important role in cancer development [15, 16]. In particular, methylation of DNA within promoter regions serves to suppress the expression of genes that can play a critical role in inhibiting tumorigenesis [17, 18]. Histone acetylation and deacetylation catalyzed by the enzymes histone acetyl transferases (HATs) and histone deacetylases (HDACs), respectively, are also important in maintaining the balance between silent and transcriptionally active chromatin. It has been shown that disruption of HATs or HDACs activity can contribute several cancers [19, 20].

In this study, based on the scenario mentioned above, it is hypothesized that down-regulation of Shelterin and Shelterin-associated genes might be under the control of DNA methylation and histone modifications. Therefore, the expression of hypermethylated genes can be actively restored after treatment with DNA methylation inhibitors, such as 5-aza-2’-deoxycytidine (5-aza-CdR). In addition, trichostatin A (TSA), a potent inhibitor of mammalian HDAC Class I and II enzymes, can be used to modify gene expression by preventing acetylation of histones within chromatin. For this reason, inhibition of histone acetylation by TSA has been used successfully as an anticancer drug [21, 22]. The effect of these drugs on Shelterin and Shelterin-associated genes was investigated in the breast cancer cell line 21NT.

Materials and methods

Cell culture conditions, reverse transcription, and quantitative real-time PCR

21NT, 21MT-2, and PB1 breast cancer cell lines were grown in modified eagle’s alpha (MEM) medium plus 10 % fetal calf serum (FCS), 1 % glutamax, 1 % HEPES, 1 % NEAA, and 1 µl/ml epithelial growth factor (EGF). GI101, BT-20, HS578-T, BT474, MTSV1-7, and MCF-7 cells were cultured in DMEM/F12 containing 10 % FCS and 1 % gluta-max, 0.5 µg/ml hydrocortisone whereas HCC1143, LY-R and LY-S were grown in RPMI/1640 plus 10 % FCS and normal mammary epithelial cell strain (LONZA) were grown in MEGM medium with recommended supplements (Table 1). All cell lines were cultured in fully humidified incubators set at 5 % CO2 and 37°C. RNA was extracted from cells using an RNasea Mini kit (QIAGEN) and cDNA was synthesized from 1 µg total RNA using Superscript reverse transcriptase enzyme (Invitrogen). Quantitative real-time reverse transcriptase PCR (qRT-PCR) was performed on the 7900HT Fast Real Time PCR machine and 1 x SYBR® green master mix (Applied Biosystems) that include 5 µM primers (Table 2) and 1 µl of cDNA.

DNA isolation and methylation-specific PCR

Genomic DNA was extracted, using the Wizard™ Genomic DNA Kit and protocol (Promega). Methylcode™ Bisulphite conversion Kit (Invitrogen) was used for bisulphite treatment of DNA samples. Methylation-specific PCR (MSP) analysis was performed for POT1 using primers (Table 3) specific for methylated and unmethylated DNA products. The MSP products were run on 2 % agarose gels and visualized using ethidium bromide (0.5 µg/ml).

Cell lines, maintenance, treatment, cell viability, and qRT-PCR

To determine the optimal drug dose, 3 x 105 21NT cells were seeded into 6-well plate. Cells that were treated with
5-aza-CdR (5 μM, Sigma, St. Louis, MO) and TSA (Sigma) (5, 15, 25, 50, and 100 ng/ml) were stained with Trypan blue (Invitrogen). Stained blue cells were scored as dead cells and unstained bright cells as viable cells. The cell viability was determined by the following formula: No. of viable cell/Total no. of cells × 100. TSA at 50 ng/ml was judged as being the best concentration to use in all experiment. The 21NT cell line was plated at a density of 1 × 10^5 in p100 culture dish, 24 h after plating; the cells were randomly assigned into treatment and control groups. Treatment included 5-aza-CdR (5 μM) for 48 h, 72 h, 3 weeks and 2 months, either alone or in combination with TSA (50 ng/ml). TSA was added only during the last 16 h of treatment. For control group, one plate was treated with 0.02 % DMSO. The short-term (48 and 72 h) and long-term (3 weeks and 2 months) effects of the drugs on the mRNA levels of Shelterin and Shelterin-associated genes were assessed using qRT-PCR in order to compare the difference between each time points of the treatment.

| Cell lines | Patient age | Histopathological diagnosis | Tumor stage | Primary site | Growth media | References |
|------------|-------------|-----------------------------|-------------|--------------|--------------|------------|
| 21NT       | 36          | PDC                         | Primary     | Breast       | MEM          | [35]       |
| 21MT-2     | 36          | PDC                         | Metastatic  | Breast       | MEM          | [35]       |
| GH101      | 57          | IDC                         | III         | Breast       | DMEM/F12 without hydrocortisone | –          |
| BT-20      | 74          | IDC                         | –           | Breast       | DMEM/F12 without hydrocortisone | [36]       |
| HS578-T    | 74          | IDC                         | –           | Breast       | DMEM/F12 without hydrocortisone | [37]       |
| BT474      | 60          | IDC                         | –           | Breast       | DMEM/F12 without hydrocortisone | [38]       |
| MCF-7      | 69          | IDC                         | IV          | Pleural effusion | DMEM/F12 without hydrocortisone | [39]       |
| HCC1143    | 52          | PDC                         | II          | Breast       | RPMI/1640    | [40]       |
| MTSV1-7    | –           | Normal immortalized mammary gland | –          | Breast       | DMEM/F12 with hydrocortisone | [41]       |
| PB1        | 36          | PDC                         | Primary     | Breast       | MEM          | [35]       |
| PC3        | 62          | Prostatic adenocarcinoma     | IV          | Prostate     | F12          | [42]       |
| LY-R       | –           | Mouse lymphoma, radiosensitive | –          | Lymphoma     | RPMI/1640    | Dr Andrzej Wojcik | University of Warszawa, Poland |
| LY-S       | –           | Mouse lymphoma, normal radiosensitive | –          | Lymphoma     | RPMI/1640    | Dr Andrzej Wojcik | University of Warszawa, Poland |
| HMEC       | –           | Normal human mammary epithelial cell strain | –          | Breast       | MEGM         | [43]       |
| Normal commercial Tissue | 78          | FirstChoice® Human breast total RNA | –          | Breast       | –            | AM6952 (Applied Biosystems) |
| PC3/hTERT  | 62          | Prostatic adenocarcinoma     | Derived from PC-3 telomerised | Derived from PC-3 telomerised | F12          | Professor Newbold group | (Brunel University) |

Table 1 Description of different cell lines and normal mammary cell strains (HMECs)

PDC primary ductal carcinoma, IDC infiltrating ductal carcinoma

Telomere length measurement by q-PCR

In order to determine telomere length, a real-time PCR technique was used. The relative telomere length was compared with that of the single copy gene (36B4 which encodes the acidic ribosomal phosphoprotein PO) [23]. Genomic DNA was extracted from 21NT-treated cells and the normal human mammary epithelial cell strain (HMEC) using the Wizard™ Genomic DNA Kit as described previously. 1× Power SYBER® Green PCR Master Mix (Applied Biosystems), 0.5 μM primes with 5 ng/μl sample DNA was used (Table 4).

Stable transfection, qRT-PCR, and western blots analysis

One day prior to transfection, 15 × 10^5 21NT cells were seeded into p100 tissue culture dishes. Cells were then transfected with the plasmid vector pcDNA-POT1/FRT/
V5-His-TOPO (a kind gift from Roger Reddel) and empty vector (pcDNA3.1/hygro plasmid kindly provided by Dr. Evgeny Makarov) using GeneJuice/C210 Transfection Reagent (Novagen) according to the manufacturer’s protocol. This vector contains hygromycin for selection in mammalian cells for stable transfection. POT1 overexpression was quantified using qRT-PCR (mRNA) and western blot (protein) analysis. For western blot analysis, cell pellets were lysed on ice using RIPA (radioimmunoprecipitation assay buffer, Sigma) buffer containing 19 protease inhibitor (Thermo). Protein concentrations were measured using the Pierce™ BCA Protein Assay Kit (Thermo Scientific). Samples (prepared in 4× Laemmle buffer containing 1.5 M Tris–Cl pH 6.8, glycerol, β-mercaptoethanol, SDS, 1 % bromophenol blue) containing 50 μg proteins were separated on a ready-made 12 % precast acrylamide gel (Bio-Rad) and were blotted onto ready-made Mini PVDF transfer membrane (Bio-Rad) using the Trans-Blot™ Turbo™ apparatus (Bio-Rad) according to the manufacturer’s protocol. The membrane was hybridized against human POT1 (1:5000, Abcam) and β-actin (1:10,000, Sigma) antibodies and the signals were detected by ECL plus (enhanced chemiluminescence) kit (GE Healthcare).

Telomerase enzyme activity

All transfected cell pellets were re-suspended in CHAPS lysis buffer (TRAPEZE® 1× CHAPS Millipore Company). The protein concentration of samples was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer’s protocol. Reaction mixtures for each of the samples to be analyzed were made up using the following components: 1× standard iTaq™ Universal Syber®-green (BIO-RAD), 0.05 μg/μl of anchored primer (ACX primer) (5’-GGCGGG (CTTACC) 3CTAACC-3’), 0.1 μg/μl of telomerase primer (TS Primer) (5’-AATCCCGTCAGCCAGTT-3’), and 250 ng/μl of protein samples. The reaction mixture was first incubated at

| Gene/assay | Forward primer (5′→3′) | Reverse primer (5′→3′) | Product size (bp) |
|------------|-------------------------|------------------------|-------------------|
| POT1-M     | AGAAAGGTTTTGTATAGGAGT   | CCAATAACTTCCAACCTTGA   | 118               |
| POT1-U     | AAGGGTTTTGTATAGGAGTTT   | CCAATAACTTCCAACCTTCA   | 116               |

M methylated, U unmethylated
25°C for 20 min to allow the telomerase in the protein extracts to elongate to the TS primer by adding TTAGGG repeat sequence. PCR was then started at 95°C for 10 min to activate Taq polymerase followed by a two-step PCR amplification of 35 cycles at 95°C for 30 s and 60°C for 90 s. Telomerase activity is reported as standard cell equivalents. To quantify telomerase activity, a standard curve was generated from serially diluted telomerase-positive prostate cell line; PC-3/hTERT (106-102). All real time amplification was carried out using an ABI Prism 7900HT (Applied Biosystems).

Results

Expression of Shelterin and Shelterin-associated genes in breast cancer cell lines and normal breast tissue

Maintenance and regulation of chromosome ends, at the telomere is fundamental for genome stability. The relationship between telosomal DNA-binding proteins and telomere length maintenance has become a popular area of interest recently. There have been a limited number of findings looking at the relationship between telomere length and Shelterin genes in breast cancer. Salhab et al. (2008) demonstrated an increase in the mRNA levels of TNKS1, hTERT, EST1, and TEP1 in breast cancer tissues whereas TNKS2 and POT1 were down-regulated in comparison with normal breast tissue [7]. Our group reported that POT1, TRF1, TRF2, SMG6, TIN2, TEP1, TNKS2, and RAP1 were significantly down-regulated at the mRNA level in breast cancer cell lines (MCF-7, GI101, BT474, 21MT-2, MTSV1-7, HCC1143, BT20, PB1, HS578-T, and 21NT) a prostate cancer cell line in comparison with RNA extracted from normal mammary breast tissue (P < 0.05, P < 0.01 and P < 0.001 correspondingly) (Fig. 1a–f and Fig. S1). However, interestingly, TPP1 mRNA levels were higher in most breast cancer cell lines compared with normal breast tissue (P < 0.05 and P < 0.01 correspondingly) (Fig. 1c).

Detection of DNA methylation of POT1 promoter region in breast cancer cell lines

Several cellular factors (i.e., mutation, DNA methylation, histone acetylation, chromatin remodeling, etc.) in theory could affect the expression of Shelterin and Shelterin-associated genes in breast cancer cell lines. Based on previous published data and our results, POT1 mRNA was found to be significantly down-regulated in malignant breast tissues and cancer cell lines [7]. Moreover, with regard to the hypothesis of a critical role of POT1 in telomere length maintenance [24], this gene was chosen for further investigation. The promoter regions of POT1 gene in breast cancer cell lines were analyzed to investigate whether methylation was responsible for the down-regulation observed. MSP primers were then designed to look for evidence of DNA methylation in the promoter of POT1 in the normal mammary cell strains (HMEC) and the breast cancer cell lines. As evident in Fig. 2a, the POT1 promoter region shows a partial methylation in breast cancer cell lines. MSP showed that the POT1 promoter contains more methylated DNA in 21NT, BT474, 21MT2, and PB1 in comparison with the remainder breast cancer cell lines and HMEC control.

Up-regulation of Shelterin and Shelterin-associated genes by 5-aza-CdR and TSA

In order to determine the best concentration of TSA to use on 21NT, an optimization experiment was carried out. Cells were treated with TSA between 5 and 100 ng/ml with 5 μM 5-aza-CdR for 48 h and a cell viability assay was carried out to look at cell survival. TSA at 50 ng/ml with 5 μM 5-aza-CdR reduced the cell viability to 78% (Fig. 2b), whereas the lowest percentage of cell survival was observed in cells treated with 100 ng/ml TSA and 5 μM 5-aza-CdR (64%). Moreover, cells treated with the combination of 50 ng/ml TSA and 5 μM 5-aza-CdR showed the highest Shelterin expression level (e.g., POT1) among the aforementioned concentrations. Consequently, 50 ng/ml was judged as being the best concentration to use as it gave the best trade-off between cell viability and the expression of Shelterin genes.

Table 4 Oligomers used for telomere length assay in 21NT-treated and normal cells [23]

| Oligomer name            | Oligomer sequence (5′ → 3′)                         | Amplicon size (bp) |
|--------------------------|----------------------------------------------------|--------------------|
| Telomere standard (human/rodent) | (TTAGGG)₄ ≈ 84                              |                    |
| 36B4 standard (human)     | CAGCAAGTGGAAGGTGTAATCCGTTACCGTCCACAGACAAG       | 75                 |
|                           | GCCAGGACTCGTTTAGCCGTGATAGAATG                   |                    |
| Telo-F (human/rodent)     | CGGTTGTGGGTTGTTGTTGGTTGTTGGTTGTTGGTTGGTTGGTTGGTTGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGG TTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGTTGGT
survival and expression (Fig. 2b, c). Treatment of 21NT breast cancer cell lines with 5 μM 5-aza-CdR and 50 ng/ml TSA over 48 h, lead to increased expression of POT1 and TIN2 as compared with the DMSO sample (P < 0.05) (Fig. S2). In contrast, the up-regulation of TNKS2, TRF1, TRF2, and RAP1 did not reach the statistical significance (Fig. S2). However, 21NT cells treated solely by TSA did not show up-regulation of TNKS2, TRF2, and RAP1 (Fig. S2). Treatment with 5-aza-CdR alone resulted in up-regulation of all the above genes except TRF1. Indeed, TPP1 after 48 h of treatment with 5-aza-CdR increased by 4-fold when compared with DMSO and untreated controls (P < 0.05) (Fig. 3c).

Effects of prolonged treatment of 21NT cells with 5-aza-CdR and TSA on transcription level of POT1 and TIN2

The preliminary results showed significantly increased expression of POT1 and TIN2 mRNA (P < 0.05) following 5-aza-CdR and 5-aza-CdR/TSA treatment (Fig. 3a, b). Therefore, we sought to investigate long-term treatment of 21NT cells to identify whether the expression of these genes may possibly change after longer period of exposure. We examined the expression of POT1 and TIN2 after treatment with 50 ng/ml TSA and 5 μM 5-aza-CdR for 48 h, 72 h, 3 weeks, and 2 months in 21NT cells. The results indicate that treatment of 21NT cells significantly increased transcription levels of POT1 and TIN2 upon treatment with TSA, 5-aza-CdR alone, and in combination with 5-aza-CdR/TSA after different time points. The result showed that the expression of POT1 and TIN2 after 48 h, 72 h, and 3 weeks of treatment with TSA and 5-aza-CdR/TSA was over 4-fold higher than in 21NT untreated cells (P < 0.05, P < 0.01, and P < 0.001 correspondingly). Nevertheless, up-regulation of POT1 and TIN2 after 2 months treatment did not reach the statistical significance (Fig. 3d, e).
Telomere length measurement by q-PCR following epigenetic changes to the Shelterin genes

To date, no previous investigations have reported on the possible effects of 5-aza-CdR and TSA can have on telomere length maintenance in cancer cells. Hence, the average telomere length was measured by q-PCR in treated and untreated 21NT cells, DMSO-treated (control) at different time points, in order to investigate whether the up-regulation of Shelterin genes such as \( \text{POT1} \), \( \text{TIN2} \), and \( \text{TPP1} \) has an effect on average telomere length. As shown in Fig. 3f short-term (72 h) and 3 weeks treatment of 21NT cells with 5-aza-CdR showed increase telomere lengths ranging from 4.5 to 4.7 kb in comparison with untreated and DMSO-treated controls whose telomere lengths were around 2 kb. The mean telomere length of the combination treatment of 21NT cells with 5-aza-CdR showed increase telomere lengths ranging from 4.5 to 4.7 kb in comparison with untreated and DMSO-treated controls whose telomere lengths were around 2 kb. The mean telomere length of the combination treatment of 21NT cells with 5-aza-CdR and TSA for 72 h and 3 weeks had a significant telomere length increase (Fig. 3f) to about 3.4 and 3.7 kb, respectively \((P < 0.05)\). BT474 and MCF7, which also had partial methylation of the \( \text{POT1} \) promoter, were then treated as above for 72 h. Both cell lines also showed an increase in telomere length (Fig. S3) therefore confirming that this is a common occurrence following treatment with 5-aza-CdR and TSA. To our surprise, treatment of HMEC with 5-aza-CdR and TSA resulted in a substantial increase in telomere length. This observation is consistent with recent report showing that treatment of cloned blastocysts and pigs by TSA resulted in telomere length elongation [25]. In addition, Blasco and her group reported that mouse embryonic stem cells genetically deficient in DNA methyltransferases at subtelomeric regions resulted in dramatically elongated telomere length [26].

Over-expression of \( \text{POT1} \) facilitates telomere length elongation in 21NT cells

Our data may suggest that the regulation of the Shelterin protein complex is needed to maintain telomere length regulation in breast cancer cell lines. Previous studies showed that reduction of \( \text{POT1} \) by RNAi results the loss of the 3' overhangs [27]. Furthermore, it had also been observed that over-expression of \( \text{POT1} \) in telomerase-positive cells resulted in telomere length elongation [24, 28].

In order to evaluate the effects of \( \text{POT1} \) (variation 1, full length) over-expression in 21NT cancer cells, these cells were transfected with human \( \text{POT1} \). As shown in Fig. 4a, \( \text{POT1} \) mRNA levels were elevated 3- to 4-fold in the \( \text{POT1}-1/\text{pcDNA5} \) and \( \text{POT1}-3/\text{pcDNA5} \) clones compared with non-transfected 21NT cells and empty vector controls. This difference was statistically significance when compared with the level in non-transfected 21NT cells \((P < 0.5)\). Western blot analysis confirmed overexpression of \( \text{POT1} \) in transfected 21NT cells at the protein level (Fig. 4b). In order to obtain a better and clear understanding of the exact role and function of \( \text{POT1} \) in protection and maintenance of telomeres in human breast cancer, we needed to determine whether overexpression of...
POT1 may play a role in telomere length maintenance. For this analysis, the q-PCR technique was used. The average telomere length of 21NT non-transfected and empty vector control clones ranged from 2.5 to 3 kb, while the POT1 over-expressing clones were between 4 and 5 kb. This represents an increase in telomere length of approximately 2 kb around 4 weeks after transfection (Fig. 4c). Telomerase activity within stable clones was measured in order to search for a link between telomerase activity and increased telomere elongation. The results presented in Fig. 4d, show that approximately 2-fold reduction in telomerase enzyme activity was observed in two stable clones compared with 21NT cells, non-transfected, and PC-3/hTERT controls. Our data suggested that telomere elongation by POT1 is not mediated by increased telomerase enzyme activity.

Discussion
Here, we have provided evidence that using inhibitors of methyltransferases (5-aza-CdR) has the potential to cause telomere elongation. The National Cancer Institute database reported that these epigenetic modification agents have been used as anti-cancer drugs in about 100 clinical trials [29]. The drugs have been used to treat different types of leukemia, sickle cell anemia, and β-thalassemia [30]. It

![Fig. 3](image-url)
has been shown that 5-aza-CdR has a global effect on gene expression in acute myeloid leukemia [31] therefore we can assume that 5-aza-CdR has a global effect on the expression of Shelterin and Shelterin-associated genes as well as DNA methyltransferases enzymes [29]. Our study has shown that in breast cancer, Shelterin expression can be down-regulated through epigenetic modification of DNA and histone proteins. Results obtained have shown that the use of chemotherapeutic epigenetic modifying drugs, such as 5-aza-CdR and TSA, induce and increase the expression of several Shelterin and Shelterin-associated genes in breast cancer cell lines. Up-regulation of these genes ultimately leads to an increase in telomere length. The effect of 5-aza-CdR and TSA on mammalian telomeres has not been widely reported in the literature to date making the results presented here novel. We can now speculate on how the use of therapeutic agents such as 5-aza-CdR and TSA to increase telomere lengths of cancer cells may benefit clinical outcome. Research into the role of Shelterin and telomerase in cancer has found that telomerase re-activation functions to maintain telomeres at a critically short length [32]. Telomeres in this unstable state are still prone to genetic damage via end-to-end fusions and translocations. This will have the effect of damaging the genome of the cancer cell giving rise to further clonal evolution and a more advanced disease. If drugs such as 5-aza-CdR and TSA are used to treat cancer, they could induce telomere lengthening. This may have the effect of stabilizing the

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**Fig. 4** a Over-expression of POT1 (variation 1) after transfection of 21NT cells. Data shows the average expression level of POT1 in 21NT non-transfected control, five vector control clones and two clones after POT1 transfection, relative to GAPDH. The non-transfected 21NT cells were used as the calibrator. **b** Western blots analysis of POT1 in 21NT transfected and control cell. A 12 % SDS-PAGE gel showing POT1 protein expression in transfected 21NT cells, two empty vector controls, PC-3, non-transfected 21NT and normal mammary epithelial cell strain (HMEC-p10). β-Actin was used as a loading control. B Western blots analysis of POT1 in 21NT transfected and control cell. A 12 % SDS-PAGE gel showing POT1 protein expression in transfected 21NT cells, two empty vector controls, PC-3, non-transfected 21NT and normal mammary epithelial cell strain (HMEC-p10). β-Actin was used as a loading control.
telomere and reducing the amount of genetic damage the cell will undergo thereby stopping the clonal evolution of the cancer cell population. These tumors may be more susceptible to further treatment as a result. Alternatively, lengthening the telomeres of cancer cells may give rise to chemo-resistant tumors. In our study, HMEC also showed an increase in telomere lengths upon treatment with 5-aza-CdR and TSA, this was unexpected as these cells already have long telomeres. HMEC’s are known to have residual telomerase activity [33], this together with the added drugs could be responsible for the telomere elongation observed in these cells. Targeting Shelterin protein components may be more effective that targeting telomerase [45], especially POT1 which is implicated to regulate telomere length and capping [4, 34]. We found that telomere elongation by over-expression of POT1 was not due to a direct effect of increased telomerase activity. Colgin et al. (2003) also observed this when they over-expressed POT1 in telomerase-positive cells, hence it seems likely that POT1 negatively regulates telomerase activity [24, 28, 44]. In conclusion, our results indicated that most of the Shelterin and Shelterin-associated genes were significantly down-regulated at the mRNA level in breast cancer cell lines. Further investigations showed that the promoter region of POT1 was partially methylated in breast cancer cell lines. We observed that 5-aza-CdR and TSA treatment of HMEC, 21NT, Bt474, and MCF7 lead to an increase in telomere lengths when compared to untreated controls.

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Conflict of interest The authors declare no conflict of interest.

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