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Camptothecin induces c-Myc- and Sp1-mediated hTERT expression in LNCaP cells: Involvement of reactive oxygen species and PI3K/Akt

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ABSTRACT

Camptothecin (CPT), a quinoline alkaloid isolated from *Camptotheca acuminate*, targets topoisomerase I, which is continuously expressed in cancer cells. However, the molecular mechanisms responsible for CPT-induced telomerase inhibition remain unclear. Unexpectedly, we found that CPT upregulates hTERT expression and concomitantly increases telomerase activity. However, transfection of *hTERT*-targeting siRNA had no effect on CPT-induced G_2/M phase arrest, suggesting that CPT-induced telomerase activation was not related to G_2/M phase arrest. CPT simultaneously increased Nrf2 expression and the level of intracellular reactive oxygen species (ROS), whereas pretreatment with the antioxidants *N*-acetyl-cysteine (NAC) or glutathione (GSH) strongly attenuated ROS production, which was accompanied by hTERT downregulation. Additionally, transient *Nrf2* knockdown enhanced CPT-induced ROS production and hTERT promoter activity. CPT also upregulated hTERT expression and telomerase activity by inducing c-Myc and Sp1 expression and activity. Moreover, c-Myc stimulated ROS production in response to CPT, leading to Sp1 activation, which promoted hTERT expression and telomerase activity. CPT treatment enhanced the phosphorylation of PI3K and Akt, which led to hTERT phosphorylation into the nucleus. These findings demonstrate that CPT positively regulates telomerase activity by upregulating hTERT expression and phosphorylation via the c-Myc/ROS/Sp1 and PI3K/Akt axis.

1. Introduction

Telomerase is a specialized ribonucleoprotein capable of maintaining telomeric repeats at the ends of eukaryotic chromosomes (Romaniuk et al., 2014). Telomerase activation is essential for the continuous growth of human tumors, indicating its critical role in tumorigenesis and metastasis (Ferguson et al., 2015). Cancer cells and spontaneously immortalized cells require elevated telomerase activity in order to maintain the telomeric ends of chromosomes; however, most normal somatic cells progressively lose their telomeres at each cell division. This is due to the functional inactivation of telomerase, which suggests that telomerase could be a potential target for cancer chemotherapy (Shay and Wright, 2010). The telomerase complex is composed of catalytic subunits, including human telomerase reverse transcriptase (hTERT), telomerase RNA (TR), chaperone proteins (p23 and Hsp90), and telomerase-associated proteins (TEP1) (Gomez et al., 2013). TR, TEP1, p23, and Hsp90 are ubiquitously expressed in a wide variety of cells irrespective of telomerase activity, indicating that these catalytic proteins are not promising therapeutic targets for cancer; however, numerous studies have focused on hTERT upregulation in cancer cell survival because most cancer cells show high hTERT expression, whereas normal somatic cells do not (Ferguson et al., 2015; Gomez et al., 2013; Romaniuk et al., 2014; Shay and Wright, 2010). Previous studies revealed that *hTERT* knockdown completely suppressed cancer cell growth by inactivating telomerase activity, whereas hTERT overexpression resulted in significantly decreased anti-cancer drug sensitivity (Li et al., 2015; Xue et al., 2010). Therefore, many attempts have been made to find specific hTERT-targeting drugs for cancer chemotherapy.

The hTERT promoter region is located at 1375 bp upstream of the transcription-start site and has a variety of transcription factor binding sites (Takakura et al., 1999). The region includes two typical E-boxes and several GC-boxes for binding transcription factors such as c-Myc and Sp1, respectively (Kyo et al., 2000). c-Myc directly binds the E-box and induces hTERT transcription, leading to subsequent cell proliferation (Zhao et al., 2014). In addition, c-Myc activation potentiates

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hTERT expression accompanied by FOXO3a, which recruits c-Myc to the hTERT promoter through c-Myc-mediated histone acetylation (Frank et al., 2003). Moreover, mutations of two E-boxes in the hTERT promoter block the DNA-binding activity of c-Myc, leading to a significant decrease in hTERT promoter activity and inhibit hTERT activity induced by c-Myc overexpression; however, the hTERT promoter activity is still maintained by direct *c-Myc* knockdown (Zhao et al., 2014), which suggests that besides c-Myc, other transcription factors function in hTERT promoter activity. The core promoter of hTERT also contains Sp1/Sp3 binding sites, which are necessary for hTERT expression (Cheng et al., 2015). *Sp1*-silencing completely inhibits telomerase activity by suppressing hTERT expression, leading to apoptosis (Bisson et al., 2015; Jeong et al., 2015). Taken together, c-Myc and Sp1 cooperatively upregulate the hTERT promoter; however, direct targeting of c-Myc and Sp1 would be an ineffective strategy to modulate hTERT expression in cancer therapy because these transcription factors are ubiquitously expressed and not only in cancer cells. Post-translational modification of hTERT is also required for telomerase activity through nuclear translocation by phosphorylating hTERT at Ser^{227} via the Akt pathway, which increases the binding affinity of hTERT to importin-α (Chung et al., 2012). Overexpression of hTERT reduces the basal level of intracellular reactive oxygen species (ROS) and suppresses ROS-mediated apoptosis (Indran et al., 2011). Nuclear factor erythroid 2-related factor 2 (Nrf2) binds antioxidant response elements (AREs) and stimulates the transcription of antioxidant proteins, leading to a decrease in ROS production (Kovac et al., 2015). However, it is unclear as to whether Nrf2 downregulates hTERT expression.

Camptothecin (CPT) is a potent topoisomerase I inhibitor isolated from *Camptotheca acuminate*; it binds the topoisomerase I-DNA complex, which is responsible for DNA replication (Zeng et al., 2012). Our previous results showed that CPT triggered apoptosis, which was induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and inhibited cancer cell invasion by suppressing matrix metalloproteinase-9 (Jayasooriya et al., 2014, 2015). CPT also upregulated G_2/M arrest (Jayasooriya et al., 2018). Murofushi et al. (2006) found that the highest levels of hTERT expression and telomerase activity were observed from the G_1 to S phase, which were 2- or 3-fold higher than the lowest levels in the G_0 phase. Klapper et al. (2003) reported that treatment with a topoisomerase II inhibitor increased the telomerase activity after DNA damage. No study has determined whether CPT alleviates hTERT, but the above mentioned studies verified that most DNA-targeting topoisomerases regulated cell cycle progression and telomerase activity.

In the present study, we found that CPT promotes c-Myc- and Sp1-mediated hTERT activity, which was accompanied by ROS production. Moreover, CPT strongly induces post-translational phosphorylation of hTERT through the PI3K and Akt pathways.

2. Materials and methods

2.1. Reagents and antibodies

CPT, *N*-acetylcysteine (NAC), glutathione (GSH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide, and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) were purchased from Sigma-Aldrich (St. Louis, MO) and an enhanced chemiluminescence (ECL) kit was purchased from Amersham (Arlington Heights, IL). RPMI 1640 medium, fetal bovine serum (FBS), and an antibiotics mixture were purchased from WelGENE (Daegu, Republic of Korea). SP600125 and Wortmannin were obtained from Calbiochem (San Diego, CA). Antibodies against hTERT, p-hTERT, cyclin E, cyclin B1, Sp1, nucleolin, p21, Nrf2, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against c-Myc, PI3K, p-PI3K, Akt, and p-Akt were purchased from Cell Signaling Technology (Beverly, MA). Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulin were purchased from KOMA Biotechnology (Seoul, South Korea).

2.2. Cell culture and viability assay

Human prostate cancer LNCaP cells were purchased from American Type Culture Collection (Manassas, VA). The cells were cultured at 37 °C in a 5% CO_2-humidified incubator and maintained in RPMI 1640 medium containing 10% heat-inactivated FBS and 1% antibiotics mixture. The cells were seeded (5×10^4 cells/ml) and incubated with CPT for 24 h. MTT assay was performed to determine the relative cell viability.

2.3. RNA extraction and RT-PCR

Total RNA was isolated using TRIzol reagent (GIBCO-BRL; Gaithersburg, MD) according to the manufacturer's recommendations. Genes of interest were amplified from cDNA that was reverse-transcribed from total RNA using the One-Step RT-PCR Premix (iNtRON Biotechnology, Sungnam, Republic of Korea). Primers for *hTERT* (Gene ID: 7015) sense 5′—CCG AAG AGT GTC TGG AGC AA-3′ and antisense (5′-GGA TGA AGC CGA GTC TGG A-3′), and *glyceraldehydes-3-phosphate dehydrogenase* (*GAPDH*) (Gene ID: 2597) sense 5′—CCA CCC ATG GCA AAT TCC ATG GCA-3′ and antisense 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′ were used. PCR was initiated at 94 °C for 2 min followed by 28 cycles of 94 °C for 1 min, 1 min at the annealing temperature, and 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. Annealing temperatures for *hTERT* and *GAPDH* were 58 °C and 60 °C, respectively. After amplification, the PCR products were separated on 1.5% agarose gels and visualized by ethidium bromide fluorescence on Chemi-Smart 2000 (VilberLourmat, Marine, Cedex, France).

2.4. Western blot analysis

Whole-cell lysates were prepared using PRO-PREP protein extraction solution (iNtRON Biotechnology). Cytoplasmic and nuclear protein extracts were prepared using NE-PER nuclear and cytosolic extraction reagents (Pierce, Rockford, IL). Each protein was separated on polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were developed using an ECL reagent.

2.5. Electrophoretic mobility shift assay

Transcription factor-DNA binding activity assays were carried out with nuclear protein extract. Synthetic complementary c-Myc (5′-GGA AGC AGA CCA CGT GGT CTG CTT CC-3′) and Sp1 (5′-ATT CGA TCG GGG CGG GGC GAG C-3′) binding oligonucleotides were 3′-biotinylated using the biotin 3′-end DNA labeling kit (Pierce) according to the manufacturer's instructions, and were annealed for 30 min at 37 °C. Samples were loaded onto native 4% polyacrylamide gels pre-electrophoresed for 60 min in 0.5*X* Tris borate/EDTA (TBE) buffer on ice. The DNA-protein complex was then transferred and cross-linked to the membrane at 120 mJ/cm^2. Horseradish peroxidase-conjugated streptavidin was used to analyze the transferred DNA-protein complex.

2.6. Flow cytometric analysis

Cell cycle progression, ROS production, and intracellular p-PI3K and p-Akt were analyzed by flow cytometry (Becton Dickenson, San Jose, CA).

2.7. Telomerase activity assay

Telomerase activity was measured using a PCR-based telomeric repeat amplification protocol (TRAP) enzyme-linked immunosorbent assay (ELISA) kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's specifications. In brief, LNCaP cells were

treated with CPT for 24 h and then lysed in lysis reagent. For the TRAP reaction, PCR was performed as follows: primer elongation (25 °C for 30 min), telomerase inactivation (94 °C for 5 min), and product amplification over 30 cycles (94 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s). Hybridization and ELISA were carried out following the manufacturer's instructions.

2.8. Luciferase assays

LNCaP cells were seeded at a density of 5×10^4 cells/ml and grown overnight. The cells were cotransfected with hTERT promoter and pCMV-β-galactosidase plasmid using Lipofectamine Plus regent (Life Technologies, Gaithersburg, MD) following the manufacturer's instructions. After 24 h-incubation, the cells were treated with CPT for another 24 h. Luciferase and β-galactosidase activities were assayed according to the manufacturer's protocol (Promega, Madison, WI).

2.9. Transfection of small interfering RNA (siRNA)

LNCaP cells were seeded on a 24-well plate at a density of 5×10^4 cells/ml and transfected with *hTERT*-, *c-Myc*-, or *Sp1*-specific silencing RNA (siRNA, Santa Cruz Biotechnology) for 24 h. For each transfection, 20 nM of siRNA duplex was added to the cells along with the transfection reagent G-Fectin (Genolution Pharmaceuticals Inc., Seoul, Republic of Korea).

2.10. Immunofluorescence staining and confocal microscopy

LNCaP cells were seeded on glass coverslips and incubated for 24 h at 37 °C with or without CPT. The cells were washed twice with PBS and fixed with 90% methanol at 37 °C for 30 min. The cells were then blocked in 10% normal goat serum for 1 h and incubated overnight with anti-p-PI3K and anti-p-Akt antibodies (1:200, Santa Cruz Biotechnology) at 4 °C. The primary antibody was removed by washing the membranes in PBS containing Triton-X (0.3%) followed by incubation for 1 h with Alexa 488-conjugated anti-mouse secondary antibody (1:200, Molecular Probes, Eugene, OR). Fluorescence signals were imaged using a confocal laser microscope FV1200 (Olympus Life Science, Tokyo, Japan).

2.11. Statistical analysis

The results shown in each of the figures in this article are representative of at least three independent experiments. All data are presented as the mean ± SE. Significant differences between groups were determined using an unpaired one-way ANOVA with Bonferroni's test. Statistical significance was indicated as ^{a} and ^{b}, and was considered at $p < 0.05$.

3. Results

3.1. CPT increases hTERT expression and telomerase activity, which is not associated with G_2/M phase arrest

In order to determine the effect of CPT on cell viability, LNCaP cells were treated with the indicated concentrations of CPT for 24 h and cell viability was determined by an MTT assay. As shown in Fig. 1A, CPT decreased the relative cell viability in a dose-dependent manner and a significant downregulation was observed from 2 μM. We then examined whether CPT regulated hTERT expression and telomerase activity in LNCaP cells. Treatment with CPT increased hTERT expression at both the transcriptional (*top*) and translational (*bottom*) levels in a dose-dependent manner (Fig. 1B). Luciferase assay also showed a significant increase in *hTERT* promoter activity (Fig. 1C). In addition, we found that CPT augmented telomere activity in a dose-dependent manner (Fig. 1D), suggesting that CPT enhances hTERT expression, leading to telomerase activation.

A recent study showed that hTERT regulated the cell cycle distribution through specific localization of telomerase, peaking at the mid-S phase (Tomlinson et al., 2006). Because our previous data showed that CPT induced a G_2/M phase arrest (Jayasooriya et al., 2018), we hypothesized that the CPT-induced G_2/M phase arrest might be induced by the increased hTERT expression in LNCaP cells. To investigate the function of hTERT in CPT-induced G_2/M phase arrest, LNCaP cells were transfected with *hTERT* targeting siRNA (sihTERT). Transfection with sihTERT significantly reduced the hTERT protein level, compared to that with control siRNA (siCON, Fig. 1E). As shown in Fig. 1F, CPT significantly induced G_2/M phase arrest; however, dissimilar to our hypothesis, sihTERT did not influence on CPT-mediated G_2/M cell cycle arrest (Fig. 1F). Furthermore, the protein level of cyclin E1, cyclin B, and p21 was not significantly altered upon transient knockdown of *hTERT* (Fig. 1G). These results indicate that CPT increases hTERT expression and telomerase activity, regardless of G_2/M phase arrest.

3.2. CPT-induced ROS production increases hTERT expression

Recently, our data showed that CPT increased intracellular ROS production, leading to checkpoint-induced cell cycle arrest (Jayasooriya et al., 2018); however, no studies have determined the direct relation between hTERT expression and ROS production in response to CPT. As shown in Fig. 2A, CPT significantly induced ROS production in LNCaP cells, whereas pretreatment with the antioxidants, NAC and GSH strongly abolished CPT-induced ROS production, but not completely. Using TRAP-ELISA, we also found that CPT-induced telomerase activity was significantly decreased in the presence of NAC or GSH (Fig. 2B) accompanied by downregulation of hTERT expression (Fig. 2C). Unexpectedly, the DNA-binding activity of Nrf2 was increased at 12 h after treatment with CPT and then gradually decreased (Fig. 2D). We next evaluated ROS production in the *Nrf2*-knockdown cells in the presence of CPT. Transfection of LNCaP cells with siNrf2 significantly increased CPT-induced hTERT promoter activity, compared with that in the CPT-treated group (Fig. 2E). Depletion of Nrf2 increased ROS production (Fig. 2F). These data indicate that ROS are a potent factor in CPT-induced hTERT expression.

3.3. CPT upregulates c-Myc- and Sp1-mediated hTERT expression

The core promoter regions (−181 bp) of *hTERT* contain two c-Myc and Sp1 binding sites, and c-Myc and Sp1 have been shown to upregulate telomerase activity through hTERT expression (Kyo et al., 2000). Since c-Myc and Sp1 are directly involved in hTERT expression and regulation, we determined whether CPT altered c-Myc and Sp1 activity and expression in LNCaP cells. CPT significantly increased c-Myc- (Fig. 3A) and Sp1-DNA binding activity (Fig. 3B) in a dose-dependent manner. Additionally, the levels of c-Myc mRNA (*top*) and protein (*bottom*) were markedly enhanced in CPT-treated cells (Fig. 3C). As shown in Fig. 3D, Sp1 was also upregulated at mRNA and protein levels.

Next, we evaluated the role of c-Myc and Sp1 in CPT-induced hTERT expression. As shown in Fig. 4A, sic-Myc and siSp1 transfection was effective in transiently silencing *c-Myc* and *Sp1*. Knockdown of *c-Myc* or *Sp-1* inhibited the CPT-induced hTERT promoter luciferase activity (Fig. 4B) and resulted in the reduction of CPT-induced telomerase activity in LNCaP cells (Fig. 4C). Both sic-Myc and siSp1 abrogated CPT-induced hTERT expression (Fig. 4D). Finally, we determined whether CPT increased the activity of *c-Myc* and *Sp1* by increasing ROS production. The RT-PCR result showed that *c-Myc* and *Sp1* were significantly upregulated by CPT alone. NAC abolished CPT-induced *Sp1* expression, but not that of *c-Myc* (Fig. 4E). These results indicate that CPT increases *hTERT* expression through an increase in c-Myc-mediated ROS production, resulting in Sp1 activation, which increases telomerase activity.

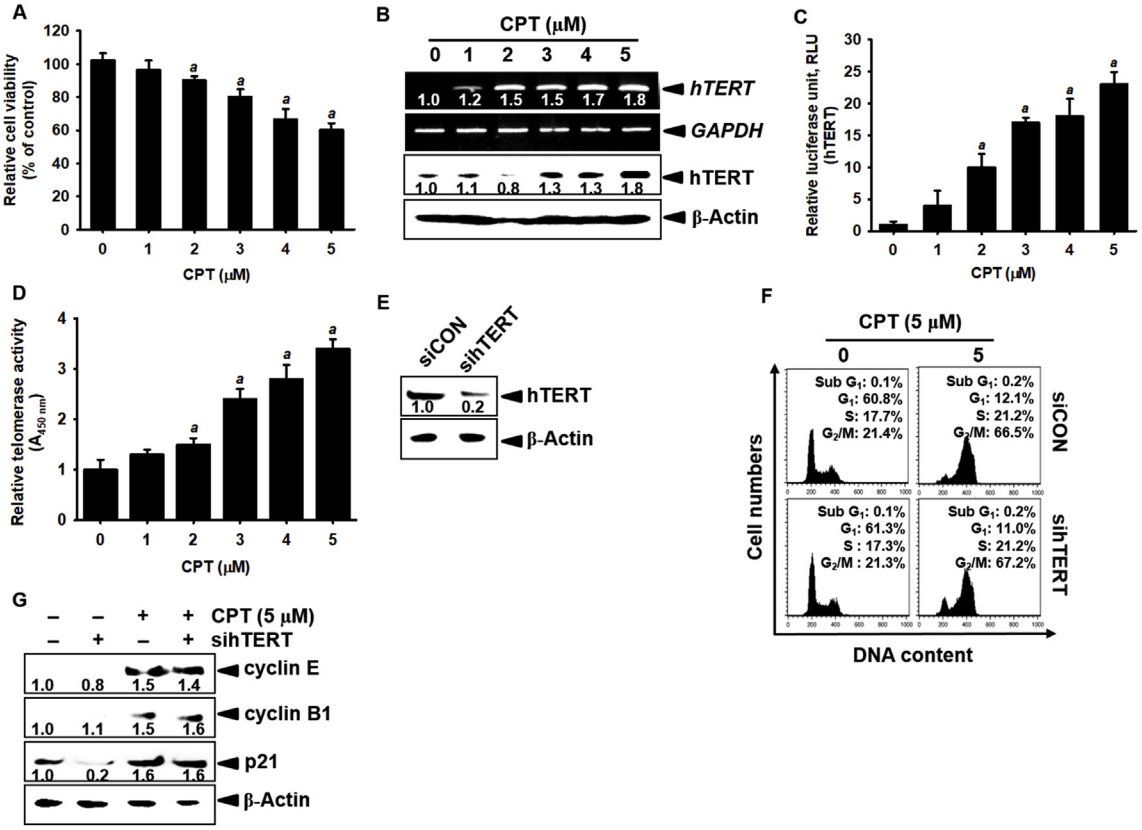

Fig. 1. Camptothecin (CPT)-induced hTERT expression and telomerase activity. LNCaP cells were treated with CPT for 24 h. (A) After a 24-h incubation, relative cell viability was determined by an MTT assay. (B) RT-PCR (*top*) and western blot analysis (*bottom*) were performed using *hTERT* primers and specific antibodies against hTERT, respectively. (C) hTERT promoter activity was measured with a luciferase assay. (D) Telomerase activity was measured by a TRAP-ELISA. (E–G) LNCaP cells were transiently transfected with *hTERT* siRNA (sihTERT) or control siRNA (siCON) for 48 h. (E and G) Western blot analysis was performed for the indicated proteins. (F) Representative cell cycle distribution was analyzed by flow cytometry. Data from three independent experiments were expressed as the overall mean ± S.E. Statistical significance was determined by one-way ANOVA (^{a}, $p < 0.05$ *vs.* untreated control).

3.4. CPT enhances PI3K/Akt signaling involved in hTERT phosphorylation

PI3K/Akt is known to enhance human telomerase activity through hTERT phosphorylation, which increases the nuclear translocation of phosphorylated hTERT (Chung et al., 2012; Jeong et al., 2015). Therefore, we determined whether CPT-induced telomerase activity was upregulated by PI3K/Akt activation. Flow cytometry data showed that CPT increased the intracellular phosphorylation of PI3K (*top*) and Akt (*bottom*) in a time-dependent manner (Fig. 5A). Western blot analysis also confirmed that CPT increased the phosphorylation levels of PI3K and Akt, although the total PI3K and Akt levels were not altered in response to CPT (Fig. 5B). Immunohistochemistry data also revealed that treatment with CPT enhanced the intensity of green fluorescence indicative of p-PI3K and p-Akt, compared to the untreated group (Fig. 5C). To further confirm the functional role of PI3K and Akt, we investigated the relation between PI3K/Akt and telomerase activity in the presence of PI3K/Akt inhibitors, LY294002 and Wortmannin. Our data showed that pretreatment with LY294002 and Wortmannin decreased the CPT-induced phosphorylation level of hTERT, which was accompanied with a significant downregulation of p-Akt (Fig. 5D). Furthermore, treatment with PI3K/Akt inhibitors significantly suppressed the CPT-induced telomerase activity (Fig. 5E). These results suggest that CPT may increase hTERT phosphorylation and thereby possibly inhibit its translocation to the nucleus by inducing Akt phosphorylation.

4. Discussion

CPT is well-known as a specific topoisomerase I inhibitor that prevents the relaxation of supercoiled DNA prior to transcription, resulting in high cytotoxic activity in a variety of cancer cell lines (Zeng et al., 2012). However, CPT has been limited in clinical trials because of its low solubility. Therefore, CPT is administrated as the sodium salt carboxylate form to increase its solubility; however, this is accompanied by poor anti-cancer efficacy due to hydrolytic sensitivity (Gottlieb et al., 1970; Hsiang et al., 1989). On the other hand, intramuscular administration of CPT completely inhibited and regressed tumor growth in human xenograft models of colon, lung, stomach, ovary, and breast cancer, without CPT hydrolysis (Giovanella et al., 1991). Nevertheless, animal models and human trials showed unpredictable toxicity such as diarrhea, dehydration, emesis, and coma (Muggia et al., 1972; Schaeppi et al., 1974). The adverse effect and poor efficacy urged the structural modification and new delivery system for CPT with an enhanced therapeutic index. Recently, Gigliotti et al. (2016, 2017) reported that CPT encapsulation by β-cyclodextrin-nanosponges significantly increased the solubility and delivery of CPT *in vivo* in xenograft tumors. This research revealed a possibility to overcome the limitation of CPT in clinical trials. Nevertheless, whether CPT activates telomerase activity has been poorly understood. In the current study, we found that CPT activates telomerase activity through c-Myc- and Sp1-mediated hTERT activation.

Telomerase activation is strongly suppressed in normal somatic cells but is reactivated in immortal cells, which suggests that upregulation of telomerase activity is crucial to the process of oncogenesis (Ferguson et al., 2015; Gomez et al., 2013; Romaniuk et al., 2014; Shay and Wright, 2010). Many studies have reported that hTERT expression, in combination with oncogenes, was sufficient to transform normal human epithelial cells and fibroblasts to tumor cells that exhibited high

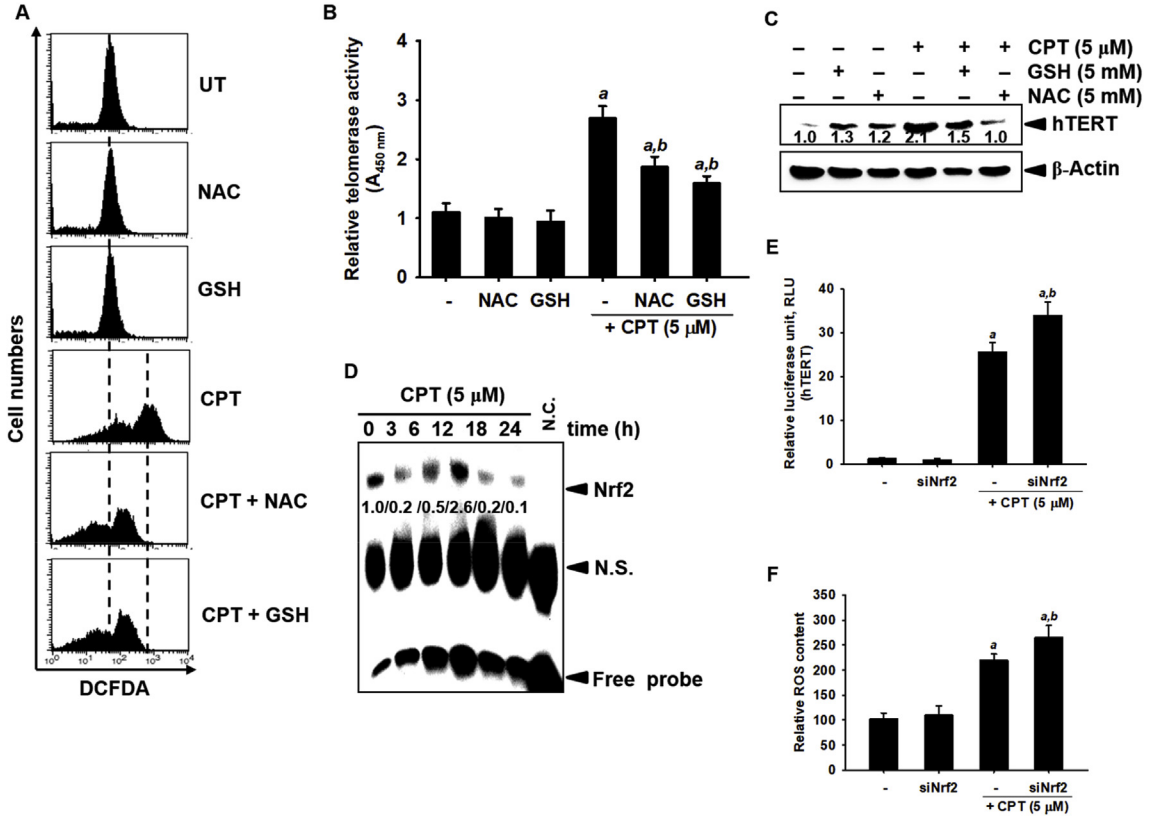

Fig. 2. ROS increases camptothecin (CPT)-induced telomerase activity. (A) LNCaP cells were treated with CPT, NAC, and GSH, or with a combination of CPT along with NAC or GSH. Intracellular ROS production was determined by the peroxide-sensitive DCFDA. (B) Telomerase activity of LNCaP cells was measured using TRAP-ELISA. (C) Western blot analysis was performed with specific antibodies against hTERT. (D) Nuclear extracts were prepared to analyze the DNA-binding activity of Nrf2 by EMSA. (E and F) *Nrf2* siRNA (siNrf2) was transfected into LNCaP cells for 48 h and treated with 5 μM DON for another 24 h. (E) The luciferase activity of the hTERT promoter was performed. (F) ROS production was detected by staining with DCFDA. Data from three independent experiments are expressed as the overall mean ± S.E. Statistical significance was determined by one-way ANOVA (^{a} and ^{b}, $p < 0.05$ *vs.* untreated control and CPT-treated group, respectively). UT: untreated, N.C.: negative control with no protein, and N.S.: non-specific.

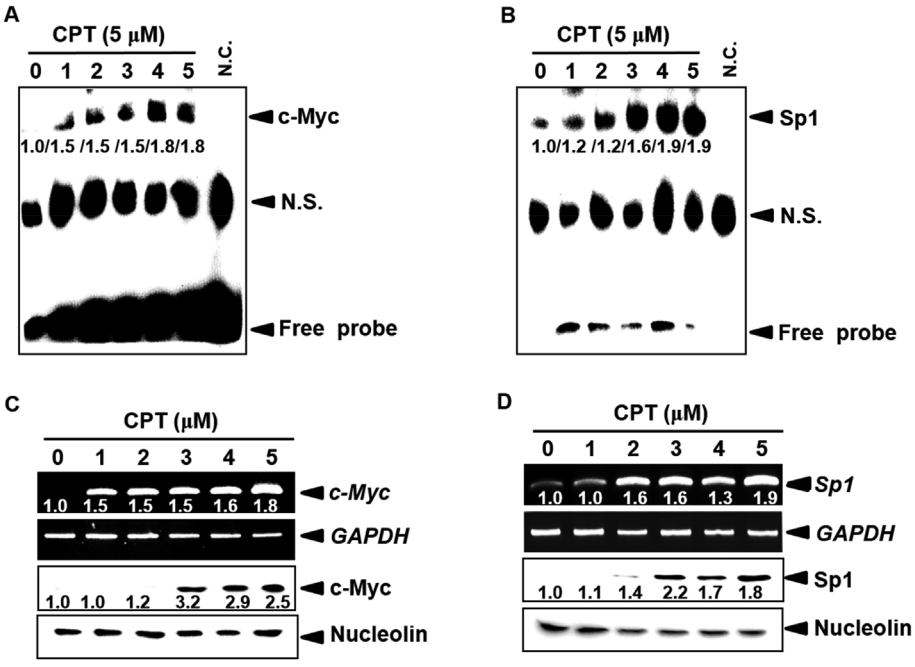

Fig. 3. Camptothecin (CPT) promotes the DNA-binding activity of c-Myc and Sp1. LNCaP cells were treated with 5 μM CPT for 30 min. (A and B) The DNA-binding activity of c-Myc (A) and Sp1 (B) was analyzed using a LightShiftTM chemiluminescent EMSA kit. (C and D) Total RNA was isolated using TRIzol reagent at 6 h and RT-PCR was performed. GAPDH was used as an internal control. Western blot analysis was performed using nuclear extracts at 24 h. Nucleolin was used as an internal loading control. Free probe refers to a probe incubated with a non-specific single-stranded DNA. N.C.: negative control with no protein and N.S.: non-specific.

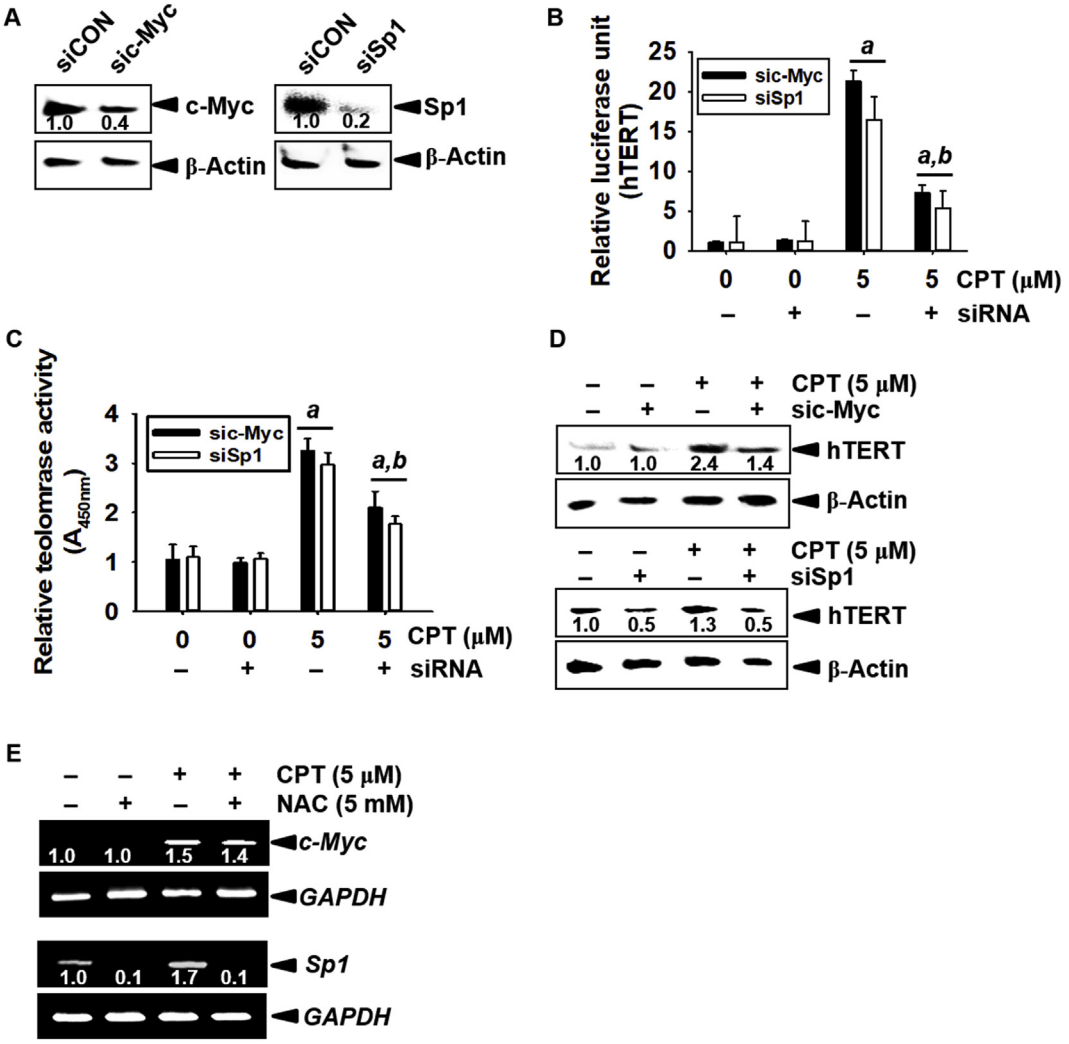

Fig. 4. Camptothecin (CPT) increases hTERT expression through c-Myc-mediated ROS production, resulting in Sp1 activation. LNCaP cells were transfected with sic-Myc and siSp1 for 48 h. (A) To check the efficiency of siRNA transfection, western blot analysis was performed for c-Myc and Sp1. (B) Cells were treated with 5 μM CPT for an additional 24 h and the promoter activity of hTERT was measured using a luciferase assay. (C) In a parallel experiment, telomerase activity was measured using TRAP-ELISA. (D and E) LNCaP cells were preincubated with 5 mM NAC for 1 h and then treated with 5 μM CPT. After 24-h incubation, western blot analysis was performed to measure the expression level of hTERT (D). Total RNA was isolated using TRIzol reagent and RT-PCR was performed at 6 h (E). Data from three independent experiments are expressed as the overall mean ± S.E. Statistical significance was determined by one-way ANOVA (^{a} and ^{b}, $p < 0.05$ vs. untreated control and CPT-treated group, respectively).

telomerase activity, enabling their uncontrolled growth (Kyo et al., 2000; Takakura et al., 1999). Telomerase has been identified as a promising target for human cancer gene therapy, as its inhibition allows telomere shortening in cancer cells, which in turn is thought to trigger apoptosis (Kailashiya et al., 2017). Murofushi et al. (2006) also reported that endogenous telomerase activity and hTERT expression could be detected in an S phase-specific manner in normal somatic fibroblasts. Liang et al. (2012) reported that immortalized human NP cells are protected against serum starvation-induced G_1 arrest via hTERT overexpression. However, in the present study, *hTERT* knockdown did not alter the CPT-induced G_2/M phase arrest, indicating that CPT increased hTERT expression and telomerase activity, which is not involved in the CPT-mediated G_2/M phase arrest. Even though another study showed that hTERT upregulation protected LNCaP cells from apoptosis (Zhang et al., 2006), we did not observe cytotoxic effects through CPT-mediated hTERT upregulation.

The hTERT core promoter contains numerous transcription factor-binding sites, including two for c-Myc, five for Sp1, one for Ets, and two for Inr (Takakura et al., 1999). Among these transcription factors, c-Myc has been studied the most extensively in the regulation of *hTERT* transcription (Rahat et al., 2014). In addition to the c-Myc recognition sequence (E-box), Sp1 is also involved in regulating hTERT promoter activity in various human cancer cells (Bisson et al., 2015; Cheng et al., 2015). In the current study, we found that c-Myc and Sp1 mRNA and protein expression was enhanced by CPT and that depletion of *c-Myc* and *Sp1* abolished the CPT-induced hTERT expression. However, we could not exclude the possibility that other transcriptional factors might also be involved in the suppression of *hTERT* expression by CPT because Ets and NF-κB are implicated in the repression of telomerase activity (Brazvan et al., 2018; Kohli et al., 2017).

According to recent research, activation of telomerase by hTERT overexpression inhibits ROS-mediated apoptosis, suggesting that ROS alleviates telomerase activity (Mattiussi et al., 2012). Additionally, Shay and Wright (2005, 2010) showed that hTERT overexpression reduces the basal cellular ROS levels as well as inhibits endogenous ROS production in response to stimuli; conversely, siRNA-mediated gene silencing of *hTERT* potentiated an increase in cellular ROS levels following exposure to oxidative stress (Indran et al., 2011). Our previous study also showed that treatment with apigenin decreased telomerase activity via an ROS-independent pathway in leukemia cells

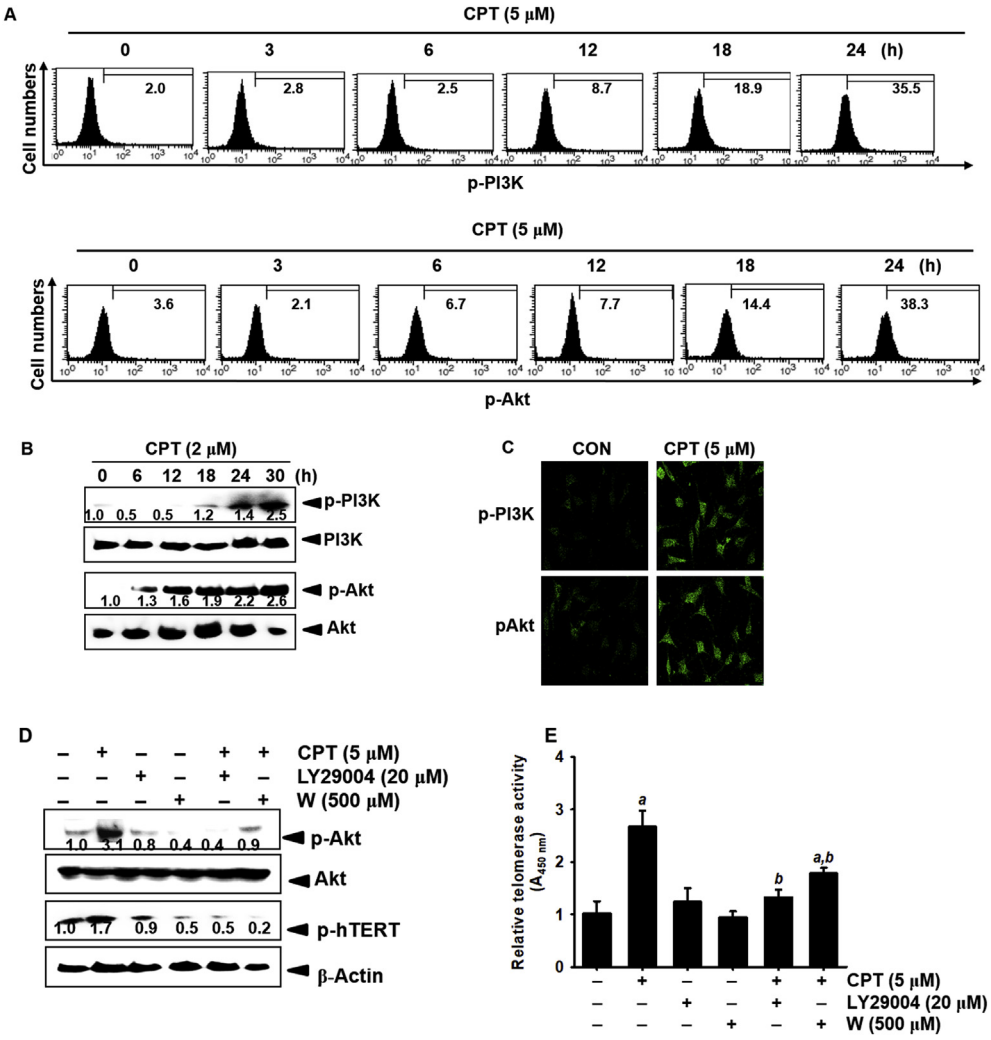

Fig. 5. Camptothecin (CPT) induces the phosphorylation of PI3K/Akt in LNCaP cells, resulting in telomerase activation. LNCaP cells were treated with CPT in the presence or absence of PI3K/Akt inhibitors such as LY294002 or Wortmannin (W). (A) The expression of p-PI3K and p-Akt was detected by flow cytometry. (B and D) Western blot analysis was performed with specific antibodies. (C) The fluorescence intensity of p-PI3K and p-Akt was observed by confocal microscopy. (E) In a parallel experiment, telomerase activity of LNCaP cells was measured using TRAP-ELISA. Data from three independent experiments are expressed as the overall mean ± S.E. Statistical significance was determined by one-way ANOVA (^{a} and ^{b}, $p < 0.05$ *vs.* untreated control and CPT-treated group, respectively). CON: untreated control.

(Jayasooriya et al., 2012). However, in the current study, we found that inhibition of ROS production decreased CPT-induced hTERT expression, indicating that ROS might increase CPT-induced hTERT expression and telomerase activation. Normally, ROS levels are tightly controlled and predominantly regulated by the transcription factor Nrf2 and its repressor protein Keap1 (DeNicola et al., 2011). Our data showed that CPT also increased the Nrf2 expression accompanied by ROS production, and that depletion of Nrf2 triggers CPT-induced hTERT expression, which is accompanied by increased ROS production. This result suggests that Nrf2 was increased as a counterpart to ROS production in the early stages, but fails to downregulate ROS-mediated telomerase activity. In addition, inhibition of ROS production suppressed *Sp1* expression, but not *c-Myc*, which means that *Sp1* expression was upregulated by CPT-mediated ROS production.

PI3K/Akt is a potent inhibitor of apoptosis that acts by blocking caspase activation and inhibiting chromatin condensation (Fulda, 2014). Akt phosphorylation could be a potent inducer for telomerase activation via hTERT phosphorylation linked to its nuclear localization (Jeong et al., 2015; Wojtyla et al., 2011). Nuclear translocation of hTERT from a presumably nonfunctional cytosolic location may be an important mechanism involved in telomerase activity in the nucleus (Kyo and Inoue, 2002). An increase in PTEN, which acts as a counterpart of Akt, may allow malignant cells to induce telomerase activity by increasing the *hTERT* mRNA levels (Zhu et al., 2014). This discrepancy implies that various kinases could possess different functions in the various signaling pathways. In the present study, we observed that treatment with CPT upregulated the phosphorylation of PI3K and Akt, leading to an increase in hTERT expression and activity. These results clearly suggest that CPT promotes hTERT at the post-translational level by inducing its phosphorylation via the Akt signaling pathway. Recently, mitogen-activated protein kinases (MAPKs) and serine/threonine kinases have been shown to phosphorylate hTERT and regulate telomerase activity (Lamy et al., 2013).

In summary, CPT enhanced c-Myc- and Sp1-mediated hTERT expression, leading to an upregulation of telomerase activity. In addition, Akt phosphorylation that is increased by CPT, enhances the phosphorylation and translocation of hTERT. This evidence suggests that CPT upregulates telomerase activity through the transcriptional and posttranslational modification of hTERT.

Conflicts of interest

The authors declare no conflicts of interest.

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