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Human Telomerase Reverse Transcriptase (hTERT): Cisplatin-내성 암의 치료를 위한 목적 단백질

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Human Telomerase Reverse Transcriptase (hTERT): A Target Molecule for the Treatment of Cisplatin-resistant Tumors

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Background : Human telomerase reverse transcriptase (hTERT) is a catalytic enzyme that is required for telomerase activity (TA) and cancer progression. Telomerase inhibition or inactivation increases cellular sensitivity to UV irradiation, DNA-damaging agents, the tyrosine kinase inhibitor, imatinib, and pharmacological inhibitors, such as BIBR1532. hTERT is associated with apoptosis. Some patients show drug-resistance during anti-cancer drug treatment and the cancer cell acquire anti-apoptotic mechanism. Therefore, we attempted to study correlation between hTERT and drug-resistance.

Methods : To study the correlation between protein level and activity of hTERT and drug-resistance, Western blotting and telomerase repeat amplification protocol (TRAP) assays were performed. To investigate whether hTERT contributes to drug resistance in tumor cells, we transiently decreased hTERT levels using small interfering RNA (siRNA) in T24/R2 cells.

Results : hTERT knockdown increased Bax translocation into the mitochondria and cytochrome C release into the cytosol. Caspase inhibitors, especially Z-VAD-FMK, rescued this phenomenon, suggesting that the stability or expression of hTERT might be regulated by caspase activity.

Conclusions : These data suggest that hTERT might be a target molecule for drug-resistant tumor therapy. *(Korean J Lab Med 2008;28:430-7)*

Key Words : *Cisplatin, hTERT, Apoptosis, Bladder cancer, Caspase*

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INTRODUCTION

Cisplatin (*cis*-diammine-dichloro-platinum^{II}) is one of the most effective chemotherapeutic agents and is widely used for the treatment of malignant tumors, including those found in the testis, head, neck, esophagus, lung, ovaries,

and bladder [1]. Cisplatin induces DNA damage, inhibits DNA synthesis, suppresses RNA transcription, blocks the cell cycle, and induces apoptosis in cancer cells [2] and cisplatin-based combination chemotherapy is a mainstay treatment for metastatic bladder cancer. However, the efficacy of cisplatin-based chemotherapy is limited by *de novo* drug resistance during chemotherapy [3]. The exact level of cisplatin resistance in patients can be inferred from clinical studies, since responses have been observed when the standard clinical dose of cisplatin was doubled in drug-intensive protocols [4]. In general, resistance to cisplatin may be substantially greater, as judged from studies of tumor cell lines established from clinically refractory tumors, which require cytotoxic concentrations as much as 50-100 fold in excess of those needed for sensitive tumor cells [5].

Telomerase is a potential diagnostic and prognostic marker in human tumors consisting of a specialized ribonucleoprotein polymerase composed of the RNA subunit, human telomerase RNA (hTR), and a catalytic protein component, human telomerase reverse transcriptase (hTERT), which can elongate telomeric DNA using its own RNA subunit as a template [6]. hTR is ubiquitously and equivalently expressed in both normal and tumor tissues, but the hTERT subunit is selectively expressed in a small subset of normal cells, tumor tissues, and tumor-derived cell lines, indicating that hTERT is the rate-limiting component of telomerase activity (TA) [7-12].

With few exceptions, TA and hTERT are found in samples obtained from bladder carcinoma, but not in normal controls. In addition, the hTR template is detected more often and at higher levels in samples obtained from bladder carcinomas than normal controls. Therefore, telomerase can identify both well-differentiated and early-stage bladder tumors [13-16]. Telomerase inhibition or inactivation increases cellular sensitivity to UV irradiation, DNA-damaging agents, the tyrosine kinase inhibitor, imatinib, and pharmacological inhibitors, such as BIBR1532 [17-20]. Telomerase inhibition is usually achieved via gene knockout, antisense oligonucleotides, or dominant-negative forms of the telomerase enzyme.

In this study, we investigated the role of hTERT in cisplatin-resistant bladder cancer cells. Acute hTERT depletion using a specific small interfering RNA (siRNA) induced Bax translocation into the mitochondrial membrane and cytochrome C release into the cytosol. Therefore, hTERT inhibition facilitates the induction of apoptosis via the mitochondrial pathway, and suggests that hTERT is critical in chemotherapy of cisplatin-resistant cells.

MATERIALS AND METHODS

1. Cell lines and culture conditions

The human bladder cancer cell line, T24, was purchased from the American Type Culture Collection (Benethda, MA, USA), and the cisplatin-resistant subcell line, T24/R2, was previously established by the stepwise exposure of T24 cells to 2 μg/mL of cisplatin [21]. These cell lines were maintained in RPMI-1640 medium (Hyclone, Road Logan, UT, USA) containing 10% FBS and antibiotics at 37℃, in a humidified incubator with an atmosphere of 5% CO_2.

2. Antibodies and reagents

Anti-hTERT and anti-caspase 3 antibodies were purchased from Calbiochem (La Jolla, CA, USA); anti-bax, anti-bcl-2, and anti-bcl-XL antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-PARP antibody from Cell Signaling Technology (Danvers, MA, USA); and anti-F1F0 ATPase and anti-alpha-tubulin antibodies from Molecular Probes Inc (Eugene, OR, USA). Anti-bak and anti-cytochrome C antibodies, a caspase-8 inhibitor (Z-IETD-FMK), and a caspase-9 inhibitor (Z-LEHD-FMK) were from BD Biosciences (Pharmingen, San Diego, CA, USA), and the general caspase inhibitor (Z-VAD-FMK) was purchased from Promega (Madison, WI, USA). Other reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated.

3. Cytotoxicity assay

The percentage of viable cells was determined using the

cell proliferation reagent, MTS (Promega). T24 and T24/R2 cells were plated in 96-well plates at 1×10^4 cells/well in 200 μL of medium. After overnight incubation, media containing cisplatin (0.1-10 μg/mL) were added. After further incubation for 24 hr, 50 μL of MTS solution was added to each well and incubated for about 30 min at 37℃. The optical absorbance at 490 nm was measured using a microplate reader.

4. Telomerase repeat amplification protocol (TRAP) PCR-based assay

TA was detected by the TRAPeze kit (Intergen, Purchase, NY, USA) according to the manufacturer's instructions. In brief, cisplatin-treated cells were lysed in 1× CHAPS (3[(3-Cholamidopropyl)dimethylammonio]-propanesulfonic acid) buffer, and the lysates were left on ice for 30 min followed by centrifugation at 12,000 rpm (14,000 g) for 20 min. Protein concentrations were measured with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), and 1.5 μg of extracts were used in the presence of an internal TRAP assay standard (36 bp) to allow quantification of activity. The telomere was extended by incubation for 30 min at 30℃ in a water bath, with products amplified by a two-step PCR (94℃ for 30 sec and 60℃ for 30 sec) for 30 cycles. TRAP products were run on a 15% non-denaturing polyacrylamide gel electrophoresis (PAGE) gel, and 6 bp telomeric ladder bands were measured by silver staining.

5. Western blot analysis

For Western blot analysis, cisplatin-treated cells were harvested and lysed in lysis buffer (20 mM Tris, pH 7.4, 2 mM EDTA, 150 mM sodium chloride, 1 mM sodium deoxycholate, 1% Triton X-100, 10% glycerol, 1 mM PMSF, 5 μg/mL aprotinin, and 10 μg/mL leupeptin). Protein concentrations were determined using the Bio-Rad protein assay kit, subjected to 8-15% SDS-PAGE, and transferred onto PVDF membranes (Hybond-P, Amersham Bioscience, Buckinghamshire, England). Subsequently, the membrane was incubated for 2 hr at room temperature in a 5% skim milk solution, and probed overnight at 4℃ with primary antibodies. The bound antibodies were visualized with a suitable secondary antibody conjugated to horseradish peroxidase using Immobilon™ Western Chemiluminescent HRP substrate (Millipore Corp, Billerica, MA, USA).

6. Synthesis of siRNA specific for hTERT and transfection

An siRNA for hTERT was chemically synthesized (Samchully Pharm, Seoul, Korea). The siRNA sequences were as follows: hTERT-siRNA (position 1,983-2,001 bp) sense, 5′-GAA CGU UCC GCA GAG AAA ATT-3′; antisense, 5′-UUU UCU CUG CGG AAC GUU CTT-3′; siRNA-control (GL2) sense, 5′-CGU ACG CGG AAU ACU UCG ATT-3′; antisense, 5′-UCG AAG UAU UCC GCG UAC GTT-3′. The transient transfection of synthetic siRNA was accomplished using Lipofectamine Plus™ reagent (Invitrogen, Grand Island, NY, USA).

7. Flow cytometry analysis of apoptosis

To measure cell death induced by cisplatin, the cells were harvested and washed with PBS one time. After fixing with 75% ethanol for 30 min at 4℃, the cells were washed with PBS three times and stained with the propidium iodide (20 μg/mL PI, 0.1 μg/mL RNase A in PBS) for 30 min in the dark. The proportion of apoptotic cells was determined by flow cytometric analysis using FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA) and Cell Quest software (Becton Dickinson).

8. Subcellular fractionation

Cytosolic and membrane-bound organelle fractions were prepared by selective plasma membrane permeabilization with digitonin. In brief, cells were exposed to 0.05% digitonin in an isotonic sucrose buffer (250 mM sucrose, 10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, and 1 mM EGTA, pH 7.1) for 5 min at room temperature to collect the soluble fraction as cytosolic extract. The digitonin-insolu-

ble fraction was washed with isotonic sucrose buffer and was further exposed to ice-cold 0.5% Triton X-100 in isotonic sucrose buffer for 10 min to collect the membrane-bound organelle fraction. Aliquots of each fraction were resolved by SDS-PAGE and were immunoblotted for bax and cytochrome C. This method routinely generates pure cytosolic and membrane fractions, as verified by immunoblotting with anti-F1F0 ATPase and alpha-tubulin antibodies.

RESULTS

1. TA and hTERT protein levels are associated with cisplatin-resistance

The T24/R2 cell line had been established by stepwise exposure of T24 cells to 2 μg/mL of cisplatin to induce resistance to cisplatin [22, 23]. We first performed MTS assays to elucidate the effect of cisplatin-induced apoptosis in T24 and T24/R2 cells. Cisplatin treatment of exponentially growing T24 and T24/R2 cells rapidly decreased the cell viability of T24 cells in a dose-dependent manner but did not affect T24/R2 cells (Fig. 1A). To test whether hTERT activity is correlated with drug resistance, we exposed T24 and T24/R2 cells to cisplatin (10 μg/mL) and measured TA, which requires hTERT. Exposure to cisplatin decreased TA in the TRAP assay of T24 parental cells but not that of T24/R2 cells (Fig. 1B) and decreased hTERT protein levels over time in T24 cells but not T24/R2 cells (Fig. 1C). Cisplatin also induced bcl-2 expression in T24/R2 cells, as previous shown [23], but not T24 cells, and did not affect the other bcl-2 family members, bcl-xL, bax, or bak. Thus, hTERT levels and TA might be involved in cisplatin resistance in T24/R2 cells.

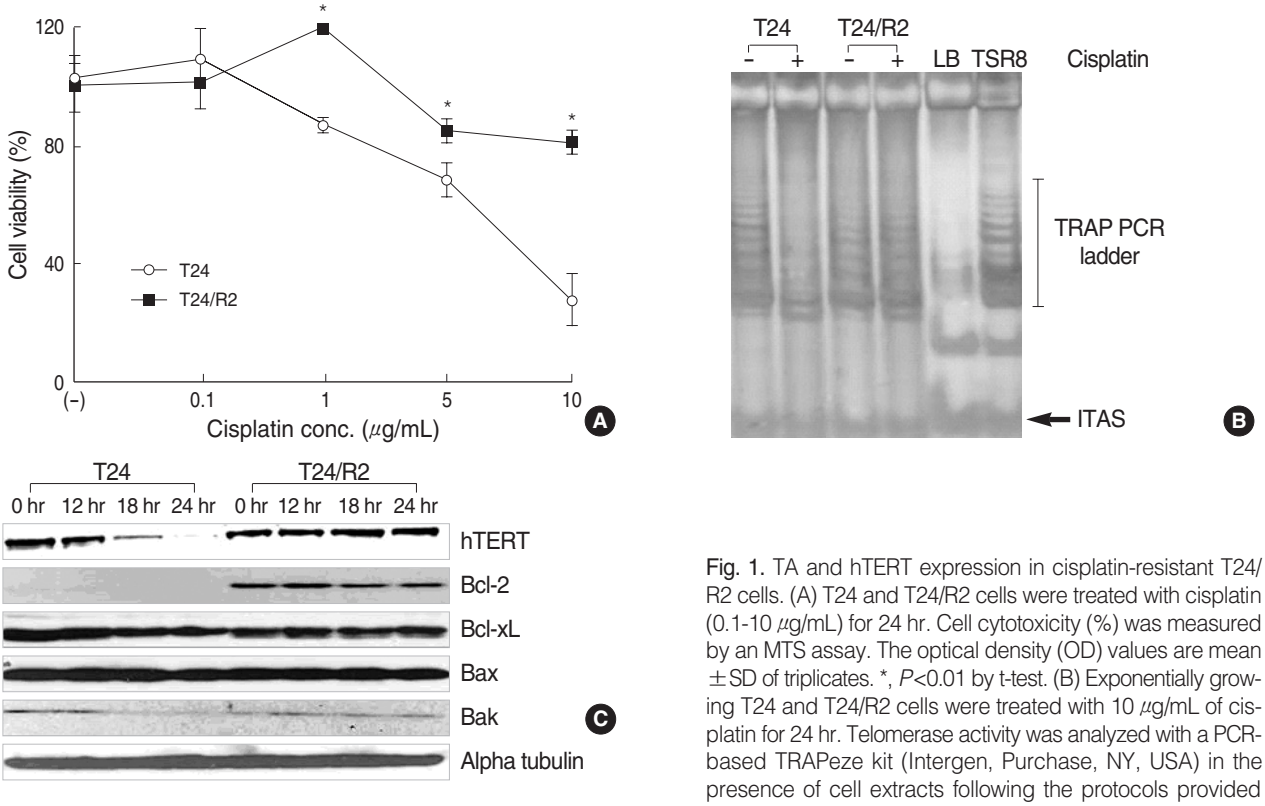

Fig. 1. TA and hTERT expression in cisplatin-resistant T24/R2 cells. (A) T24 and T24/R2 cells were treated with cisplatin (0.1-10 μg/mL) for 24 hr. Cell cytotoxicity (%) was measured by an MTS assay. The optical density (OD) values are mean ±SD of triplicates. *, $P<0.01$ by t-test. (B) Exponentially growing T24 and T24/R2 cells were treated with 10 μg/mL of cisplatin for 24 hr. Telomerase activity was analyzed with a PCR-based TRAPeze kit (Intergen, Purchase, NY, USA) in the presence of cell extracts following the protocols provided by the manufacturer. (C) T24 and T24/R2 cells were treated with cisplatin (10 μg/mL) for the periods indicated. Forty μg aliquots of cell lysate were immunoblotted with anti-hTERT, bcl-2, bcl-xL, bax, bak, and alpha-tubulin antibodies.
Abbreviations: TA, telomerase activity; hTERT, human telomerase reverse transcriptase; TRAP, a polymerase chain reaction-based telomere repeat amplification protocol assay.

2. Knock-down of hTERT accelerates Bax translocation and cytochrome C release in a caspase-dependent manner

TA is regulated by bcl-2, and overexpression of bcl-2 leads to reduced bax translocation and cytochrome C release [23, 24]. To test whether hTERT expression affected Bax translocation, we transfected T24/R2 cells with hTERT siRNA using Lipofectamin (Fig. 2A). Cisplatin treatment of cells transfected with the siRNA increased bax levels in the mitochondrial membrane fraction and cytochrome C in the cytosol compared to control siRNA cells (Fig. 2B), suggesting that hTERT depletion affects the membrane permeabilization of mitochondria via increasing bax translocation and inducing cytochrome C release, which activates the caspase cascade to induce apoptosis.

To investigate whether the caspase cascade modulated the role of hTERT, we treated si-hTERT T24/R2 cells with cisplatin in the presence of caspase inhibitors. While hTERT knock down increased membrane-bound bax and cytosolic cytochrome C, these effects were inhibited by caspase 8, 9, and particularly Z-VAD-FMK inhibitors (Fig. 3A). Caspase inhibitors also blocked the increase in cisplatin-induced apoptosis induced by hTERT deficiency (Fig. 3B).

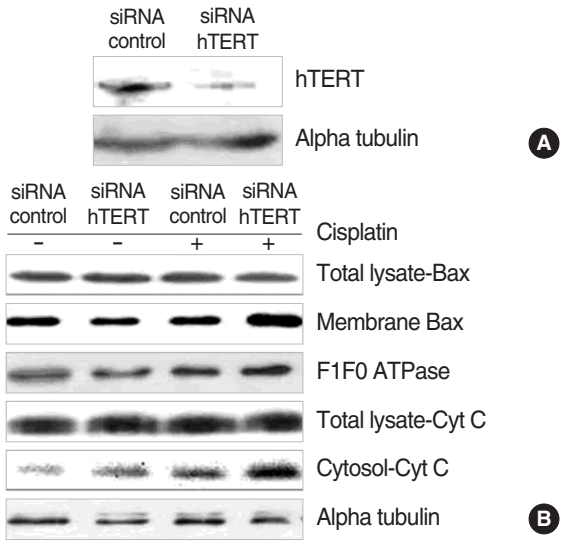

Fig. 2. Effects of hTERT knock down on bax translocation and cytochrome C release. (A) T24/R2 cells were transfected with hTERT siRNA using Lipofectamine. After 24 hr, the cells were treated with cisplatin for an additional 24 hr and western blot analysis was performed. (B) T24/R2 cells transfected with hTERT siRNA were separated into cytosolic and membrane fractions. The extracts were subjected to 15% SDS-PAGE and immunoblotted with anti-bax, cytochrome C, F1F0 ATPase, and alpha-tubulin antibodies.
Abbreviations: hTERT, See Fig. 1.

DISCUSSION

Bladder cancer is the most common malignant neoplasm of the urinary system, with increasing morbidity and mortality. Most cases of bladder cancer initially respond to cisplatin-based combination chemotherapy, but acquired resistance usually occurs during disease progression [1]. Measuring

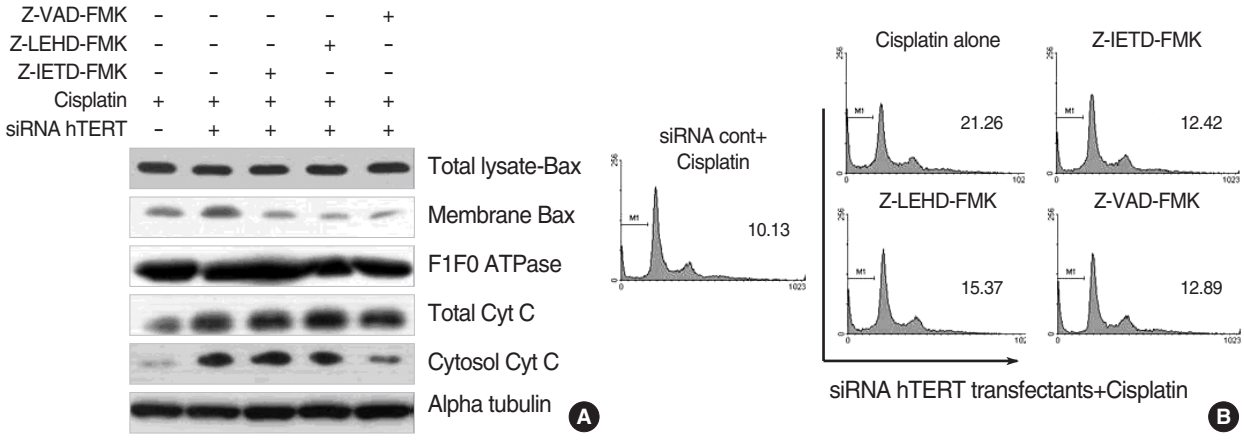

Fig. 3. Caspase inhibitors block the effects of hTERT knock down in cisplatin-induced apoptosis of T24/R2 cells. (A) T24/R2 cells were transfected with hTERT siRNA and pre-treated with caspase inhibitors before cisplatin treatment. After further incubation for 24 hr, the cells were fractionated and the locations of Bax and cytochrome C were evaluated by western blotting. (B) Treatment as in (A). Cells were fixed with ethanol and stained with PI solution in the dark for 30 min. Apoptotic cell death was determined by flow cytometry.
Abbreviations: hTERT, See Fig. 1; PI, propidium iodide.

TA in the voided urine of patients with bladder cancer may be useful in cancer diagnosis, prognosis, and monitoring of residual disease [16], since the abnormal expression of telomerase is related to the occurrence and development of malignant neoplasms. hTERT, one of the three components of telomerase, is the rate-limiting factor of TA. hTERT is expressed in over 92% of bladder cancers and may be a reasonable therapeutic target to suppress bladder cancer growth [24, 25].

Here we used a cisplatin-resistant bladder cell line to clarify the role of hTERT in cisplatin resistance. T24 parental cells showed decreased TA compared to T24/R2 cells in the presence of cisplatin. Cisplatin treatment reduced the viability of T24 cells by 80% in dose-dependent manner, but did not affect that of T24/R2 cells. Cisplatin treatment significantly reduced hTERT levels in T24 cells after more than 12 hr' exposure but not in T24/R2 cells.

The inhibition of telomerase in tumor cells represents a promising anticancer strategy, leading to telomere destabilization and consequent growth inhibition and cell death [18, 20, 26]. However, its effectiveness could be impaired by a lag phase, depending on the initial telomere length [27]. Telomerase inhibition may take weeks or months of treatment to affect tumor cell growth [26, 28-30]. However, other studies show an immediate cellular response to telomerase inhibition regardless of telomere length [31-35]. Therefore, targeting telomerase expression could regulate cell proliferation and apoptosis independent of reducing telomere length.

We next explored whether hTERT could modify mitochondrial function using an siRNA specific for hTERT and measuring the localization of bax and cytochrome C. Knock down of hTERT increased cisplatin-induced cell death and accelerated the translocation of bax into the mitochondrial membrane and the release of cytochrome C, both of which could be blocked by caspase inhibitors. hTERT has a crucial role in mitochondria during intrinsic apoptosis. Moreover, hTERT deficiency increased apoptosis through bax translocation and cytochrome C release via caspase activity. In conclusion, hTERT knock down with an siRNA increased the cisplatin sensitivity in cisplatin-resistant tumor cells, suggesting that hTERT may be a suitable target molecule for drug-resistant bladder cancers.

요 약

배경 : Human telomerase reverse transcriptase (hTERT)는 telomerase의 활성과 종양의 진행과정에 관련이 있다고 알려져 있다. Telomerase 억제 또는 비활성화는 UV 조사, DNA 손상유도 약물, 타이로신 카이네이즈의 억제제, imatinib, BIBR1832와 같은 약물에 대한 감수성을 증가시킨다. 특히 hTERT는 세포사멸기작에도 작용한다. 항암제 치료를 통하여 항암제 내성기작을 보이는 환자가 발생할 수 있으며, 그 암세포는 항암제에 대한 세포사멸에 저항적 기작을 획득하게 된다. 따라서 본 연구에서는 hTERT와 항암제 내성기작과의 관련성을 연구하고자 하였다.

방법 : hTERT 단백질 및 telomerase의 활성과 약물에 대한 내성기작의 관련성을 연구하기 위해 Western blotting과 TRAP을 하였다. hTERT가 종양세포의 약물내성에 직접적으로 관여하는지를 검증하기 위해 T24/R2 세포주에 siRNA를 사용하여 hTERT 발현을 억제하였다.

결과 : hTERT의 발현억제는 BAX의 사립체 내로의 이동을 증가시켰고, 세포질로의 cytochrome C의 방출을 증가시켰다. Z-vad-FMK와 같은 caspase의 억제제는 이러한 현상을 억제하였으며, 이는 hTERT의 안정성과 발현이 caspase의 활성에 의해 조절될 수 있음을 보여준다.

결론 : Telomerase의 구성단백질의 기능적 구성체인 hTERT는 항암제 내성세포를 대상으로 하는 치료에 있어서 강력하고 적절한 목적 단백질로 사료된다.

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