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Full Length Research Paper

Experimental therapy against ovarian cancer by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene driven by human telomerase reverse transcriptase (hTERT) promoter

Hongmei Li[1] and Yuecheng Yu[2]*

[1]Department of Obstetrics and Gynaecology, Affiliated Hospital of Yan'an University, China.
[2]Department of Obstetrics and Gynaecology, Xijing Hospital, Fourth Military Medical University, China.

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TRAIL functions as a soluble cytokine killing various cancer cells with limited toxicity to most normal cells. Human telomerase reverse transcriptase (hTERT) promoter is known to selectively drive transgene expression in many human cancer cells. In this study, we aimed to determine whether the hTERT promoter could efficiently mediate more specific TRAIL gene therapy in ovarian cancer cells. We demonstrated that green fluorescent protein (GFP) expression driven by the hTERT promoter was observed in several ovarian cancer cells, but not in normal epithelial cells. hTERT promoter activity was comparable to that of Cytomegalovirus (CMV) promoter in ovarian cancer SKOV3 cells, as judged by GFP expression level detected by real-time polymerase chain reaction (PCR) and flow cytometry, as well as TRAIL expression detected by western blot. Both hTERT-TRAIL and CMV-TRAIL constructs displayed comparable proliferation inhibition and induced cell apoptosis in ovarian cancer SKOV3 cells, and both exerted effects that are statistically different from control group. Moreover, *in vivo* studies indicated that intratumoral administration of these vectors significantly suppressed the growth of xenograft tumors, compared with control group. These data demonstrated that hTERT promoter can efficiently and specifically drive TRAIL gene expression in ovarian cancer SKOV3 cells, thus specifically induce apoptosis in these cells and reduced cancer growth *in vivo.*

Key words: Human telomerase reverse transcriptase (hTERT), promoter, tumor necrosis factor related apoptosis-inducing ligand (TRAIL), gene therapy, ovarian cancer.

INTRODUCTION

As the fifth leading cause of cancer deaths among women, ovarian cancer has the highest mortality rate of all gynecologic cancers (Jemal et al., 2009). Despite the advances in diagnostic procedures, surgical techniques, radiation therapy, and chemotherapy, the 5-year survival rate for late-staged patients is still below 25% (Matei, 2007; Ozols et al., 2003). Thus, it is crucial that more effective and/or alternative therapeutic strategies are developed to treat ovarian cancer.

Decreased sensitivity to apoptotic signals as well as of enhanced cell proliferation are believed to be the direct cause of cancer progression (McDonnell et al., 1995; Thompson, 1995). Therefore, a genetic restoration of apoptotic pathway or introduction of apoptosis-inducible gene might be an attractive approach for the treatment of cancer, including ovarian cancer. Tumor necrosis factor related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor family (Wiley et al., 1995; Sheridan et al., 1997). After binding to the death domain-containing receptors, TRAIL initiates the apoptotic signal

*Corresponding author. E-mail: yuych2006@yahoo.com.

inside the cells (Kuang et al., 2000). However, its widely application was hampered due to its systemic cytotoxicities.

To succeed in cancer gene therapy, the efficient delivery of therapeutic genes to a target site is a major challenge. Recombinant viral vectors account for the majority of gene delivery approaches used in current cancer gene therapy studies. Though effective, there are concerns for safety issue due to immunogenicity of viral proteins, risk of oncogenesis and inadvertent creation of infectious viral particles (Russ and Wagner, 2007). Plasmid DNA, on the other hand, is a relatively safe alternative to viral vectors. Cytomegalovirus (CMV) early promoter is a well-known powerful promoter frequently used for driving the expression of transgenes in mammalian cells (Laddy and Weiner, 2006). However, its ubiquitous expression without tumor-targeting activity, limited its applications.

Telomerase is a distinctive candidate for targeted gene therapy of cancer (Nakamura et al., 1997; Kilian et al., 1997). It is active in 85 to 90% of cancer cells with its activity correlates well with the degree of malignancy (Wright et al., 1996; Shay and Bacchetti, 1997; Kim et al., 1994). The selective expression of telomerase in cancer cells versus normal cells is largely due to transcriptional activation of the human telomerase reverse transcriptase (hTERT) gene (Gunes et al., 2000). A number of studies have demonstrated that the hTERT promoter is very effective in driving specific gene expression in tumor cells while its activity in normal cells is generally undetectable (Koga et al., 2000; Gu et al., 2000). Thus, the use of hTERT promoter constructed expression vector is an ideal choice for specific gene therapy.

In ovarian cancer, hTERT promoter had been used to drive the expression of HSV-TK (herpes simplex virus thymidine kinase) (Song et al., 2003) and tumour necrosis factor (Murugesan et al., 2007), but few work on TRAIL under the regulation of hTERT promoter had been reported. In previous study, we generated a eukaryotic expression vector, in which the expression of TRAIL is driven by hTERT promoter (Li et al., 2007). In order to compare gene expression specificity and efficacy, we also generated a eukaryotic expression vector, in which the expression of TRAIL is driven by CMV promoter. We hypothesized that hTERT promoter could efficiently regulate the expression of TRAIL, thus specifically induce apoptosis of cancer cells. To test this hypothesis, we investigated the *in vitro* and *in vivo* functional effects of the aforementioned constructs using ovarian cancer SKOV3 cells in this study. We initially studied the effects of these constructs through a number of *in vitro* assays, such as cell growth inhibition, TUNEL assay, and soft-agar colony-forming assay. Then by *in vivo* athymic nu/nu mouse model, we assessed their effects on xenograft growth established via subcutaneous transplantation of human ovarian cancer cells. Our results indicated that hTERT-TRAIL construct is a specific and potent antitumor gene therapy strategy against ovarian cancer.

MATERIALS AND METHODS

Cell culture and reagents

Human endothelial cell line, ECV304 , human ovarian cancer cell lines SKOV3, HO-8910, and human cervical cancer Hela cells preserved in Xijing hospital were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin.

Cell transfection

Cells were plated in 6-well plates at density of 1.5×10^5 cells/well, respectively. hTERT-TRAIL, CMV-TRAIL, hTERT-EGFP (enhanced green fluorescent protein) and CMV-EGFP constructs were transfected into cells separately. Transfection was performed using Lipofectamine^{TM} 2000 kit (Invitrogen) according to the instructions of manufacture. After the transfection for 48 h, cells were harvested for analysis. To observe GFP expression, 48 h after transfection, cells were visualized by fluorescence microscopy to detect GFP expression in cancer cells or normal epithelial cells respectively, or quantified by flow cytometry.

Quantitative real-time PCR

After transfection for 48 h, total cellular RNA was isolated using TRIzol reagent (Life Technologies, USA) according to the manufacturer's protocol. GFP primers and probes were obtained from Takara Bio Inc. (Madison, Wisconsin, USA) and the hTERT gene expression assay was performed according to the instruction from the manufacturer. The RT-PCR reaction was conducted using approximately 50 ng of total RNA. The reaction was conducted under default RT-PCR conditions at 48°C for 30 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and at 60°C for 1 min. Relative quantitation of gene expression between multiple samples was achieved by normalization against endogenous GAPDH RNA (primer and probe from ABI, Foster City, CA) using the $\Delta\Delta C_T$ method of quantitation provided by the manufacturer.

Western blot analysis

After 48 h, cells were transfected by different vectors; the cells were washed with phosphate-buffered saline (PBS) and collected with the cell scraper. Total cell lysates were prepared in extraction buffer as described (Yuecheng and Xiaoyan, 2007). After electrophoresis using 12% SDS-PAGE, samples were transferred onto nitrocellulose membranes (Millipore). The membrane was blocked for 1 h at room temperature in PBS containing 5% powdered milk and then was incubated with Mouse anti-TRAIL monoclonal antibody (1:1000) overnight at 4°C. The secondary antibody was horseradish peroxidase-conjugated antimouse IgG antibody. The membranes were reacted for 5 min with SuperSignal west pico chemiluminescent substrate (Pierce). Each experiment was

repeated three times, with similar results. Individual bands on western blots were quantified using AlphaEaseFCt software (Alpha Innotech Corporation, San Leandro, CA, USA). All data were within the linear range of detection for each antibody used.

Cell proliferation assay

The cells (2×10^4 / ml) were plated in 96-well plates and 24 h later transfected as described. 24, 48 and 72 h later, cells were treated with 10% 3-(four, 5-dimethylthiazol-2-yl)-two, 5-diphenyltetrazolium bromide (MTT) reagent for 1 h and then evaluated colorimetrically at 575 nm using the Labsystems Multiskan MS Version 3.0 (Helsinki, Finland). Three separate experiments were performed and each assay was conducted in triplicate.

Colony-formation in soft agar assay

To further evaluate cell survival and proliferation, we performed a colony-formation assay. A bottom layer of 0.5 × DMEM media + 0.5% agar was poured into 1.5 ml/ 3.5 cm Petri dishes. After the agar solidified, a top layer of 0.5 × DMEM media + 0.35% agar mixed with a specified number of transfected cells (5000 cells/dish) was added to the dish. The dishes were incubated at 37°C in a humidified incubator for 14 days. Colonies were stained with 0.5 ml of 0.005% crystal violet for at least 1 h, and then counted using a dissecting microscope.

TUNEL assay

To detect the induction of apoptosis, 48 h after transfection, cells were fixed with 1% formaldehyde and 0.2% glutaraldehyde for 5 min, rinsed with phosphate-buffered saline (PBS), and stained with the terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end-labeling (TUNEL) technique (ApopTag®Peroxidase *in situ* Apoptosis Detection Kit, Intergen, Purchase, NY). Cells were visualized by either bright field or fluorescence microscopy to detect apoptotic cells or GFP positive cells, respectively. An apoptotic index was determined as a percentage of apoptotic cells among 100 GFP-positive cells.

In vivo evaluation of antitumor effect

To determine the antitumor effect of TRAIL driven by hTERT or CMV promoter *in vivo*, 40 athymic female BALB/c nu/nu mice, 4 to 6 week of age per group, were hosts for human orthotopic xenograft tumors. All mice were housed in a specific pathogen-free environment in compliance with institutional policy, and all animal procedures have been approved by the appropriate institutional review boards. SKOV3 cells (2×10^6) suspended in 100 µl of serum-free DMEM were inoculated subcutaneously and bilaterally into female BALB/c nu/nu nude mice 4 to 6 weeks old. Endotoxin-free plasmids were purified by Qiagen Endo-Free Mega Plasmid kit (Qiagen) according to a commercial protocol. Using a chromogenic Limulus amoebocyte clotting assay kit (QCL-1000, BioWhittaker), the endotoxin level was determined to be <10 endotoxin units/mg of DNA. At 10 days, when most of the tumors had reached 3 to 4 mm in diameter, the mice were randomly divided into four groups: hTERT-TRAIL, CMV-TRAIL, hTERT-EGFP and CMV-EGFP, and intratumoral Lipofectamine-mediated transfer of these constructs was performed three times (days 10, 14 and 18). Tumor growth was monitored using calipers every 4 days for six consecutive weeks after the beginning of the injection. The tumor volumes (mm^3) were calculated as length X $width^2/2$. The changes of tumor volume were calculated by subtracting the tumor size during the first injecting time from final tumor size.

Statistical analysis

Data in this paper are expressed as mean ± SEM. Student's t-test was used to compare the data between control and treated cells, and $p< 0.05$ was taken as the level of significance. All results were analyzed by statistical software SPSS10.0.

RESULTS

GFP gene expression driven by hTERT promoter is specifically expressed in ovarian cancer cells

For target gene therapy, it is essential that death-inducing genes be expressed only in cancer cells, but not in normal cells. To compare the gene expression efficacy and specificity, we first set out to test the gene expression under the control of hTERT promoter or CMV promoter in a panel of ovarian cancer cell lines (SKOV3, HO-8910), human epithelial cervical cancer cells (Hela) and normal cells (normal ovarian epithelial cells ECV304). Observed under fluorescence microscopy for GFP expression, we found that GFP expression driven by hTERT promoter is at the similar level as that driven by CMV promoter. Most importantly, no GFP expression driven by hTERT promoter in normal epithelial cells was observed, suggesting that the promoter remained inactive in normal cells (Figure 1A). Further quantity study for GFP gene expression efficacy was done by real-time PCR and demonstrated similar GFP expression level driven by hTERT promoter and CMV promoter in cancer cell lines (Figure 1B). Our data demonstrated that hTERT promoter would be an excellent choice as an ovarian cancer specific promoter to express target gene that could be applied to gene therapy.

Gene expression level is comparable driven by hTERT promoter or CMV promoter in ovarian cancer cell SKOV3

For gene therapy to be efficacious in the treatment of cancer, therapeutic genes need not only be expressed specifically inside cancer cells, but also be expressed at sufficiently high transcriptional levels. We next compare the gene expression level driven by hTERT promoter or CMV promoter in ovarian cancer cell SKOV3. SKOV3 cells were transfected using liposome transfection method with TRAIL or GFP under the control of hTERT promoter or CMV promoter respectively, and were named

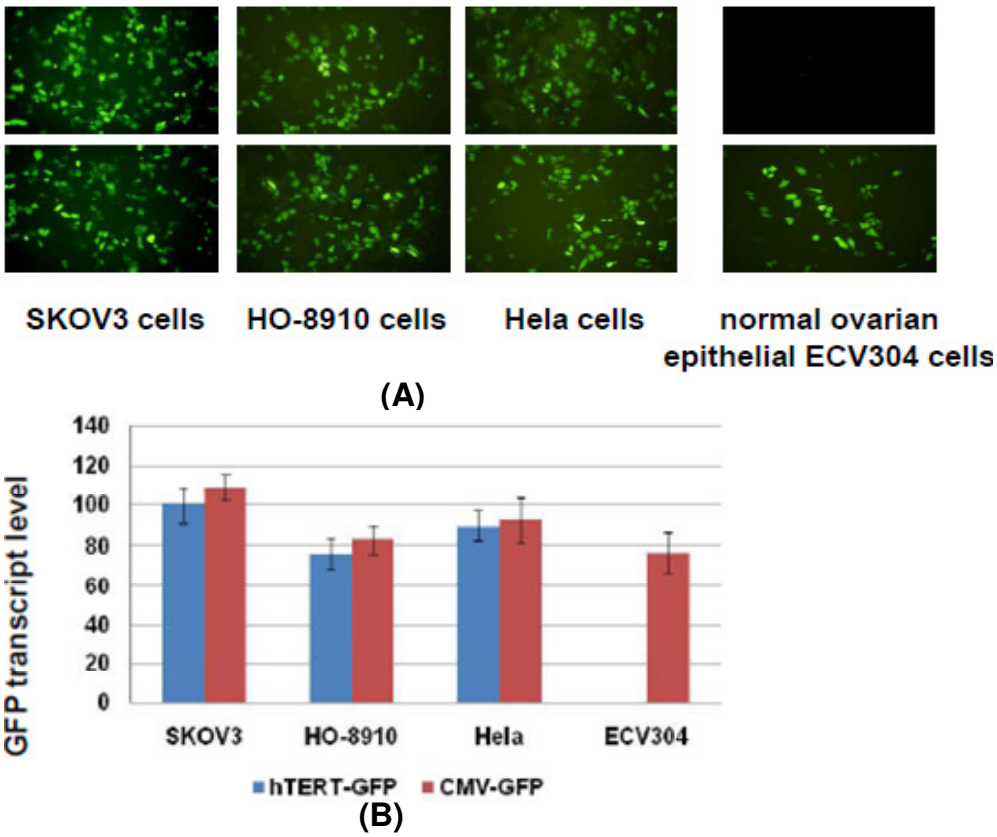

Figure 1. GFP expression specificity and efficiency in cancer cells and normal cells driven by hTERT promoter and CMV promoter. Two ovarian cancer cell lines (SKOV3, HO-8910), normal ovarian epithelial cell line (ECV304) and cervical cancer cell line (Hela) were transiently transfected with hTERT-GFP (upper panel) or CMV-GFP plasmids (lower panel). 48 h after transfection, (A) GFP expression were visualized by fluorescence microscopy or (B) RNA were extracted and GFP transcript levels were measured by quantitative reverse transcription-polymerization chain reaction (qRT-PCR) and normalized to that of GAPDH. The GFP transcript level in SKOV3 cell was arbitrarily set to 100. All results are representative of two independent experiments.

as SKOV3-hTERT-TRAIL, SKOV3-CMV-TRAIL, SKOV3-hTERT-GFP and SKOV3-CMV-GFP. First, we detected GFP expression by flow cytometry. As shown in Figure 2A, in SKOV3-hTERT-GFP, fluorescence expression rate is 55.2%; while in SKOV3-CMV-GFP, fluorescence expression rate is 59.7%. No significant difference existed between two promoters. Next, western blot was used to measure TRAIL protein expression in SKOV3 cells after transfection. About 3.6 fold of TRAIL expression were attained in SKOV3-hTERT-TRAIL and SKOV3-CMV-TRAIL cells, as compared to that in SKOV3-hTERT-GFP and SKOV3-CMV-GFP cells (Figure 2B). All together, our results demonstrated specific and efficient gene expression driven by hTERT promoter, as compared with that by CMV promoter.

TRAIL expression under hTERT promoter inhibited ovarian cancer cell growth

We next examine the functional effects of *in vitro* cell growth inhibition by TRAIL gene driven by hTERT promoter or CMV promoter in ovarian cancer cell line SKOV3. As shown in cell growth curves (Figure 3A), there were significant inhibition of cell proliferation in SKOV3-hTERT-TRAIL and SKOV3-CMV-TRAIL group, while there was no inhibition in SKOV3-hTERT-GFP and SKOV3-CMV-GFP cells ($p<0.05$). Soft-agar colony formation assay was also used to test the effects of TRAIL expression in ovarian cancer cells. As Table 1 showed, the colony formation rate in SKOV3-hTERT-TRAIL and SKOV3-CMV-TRAIL group were significantly

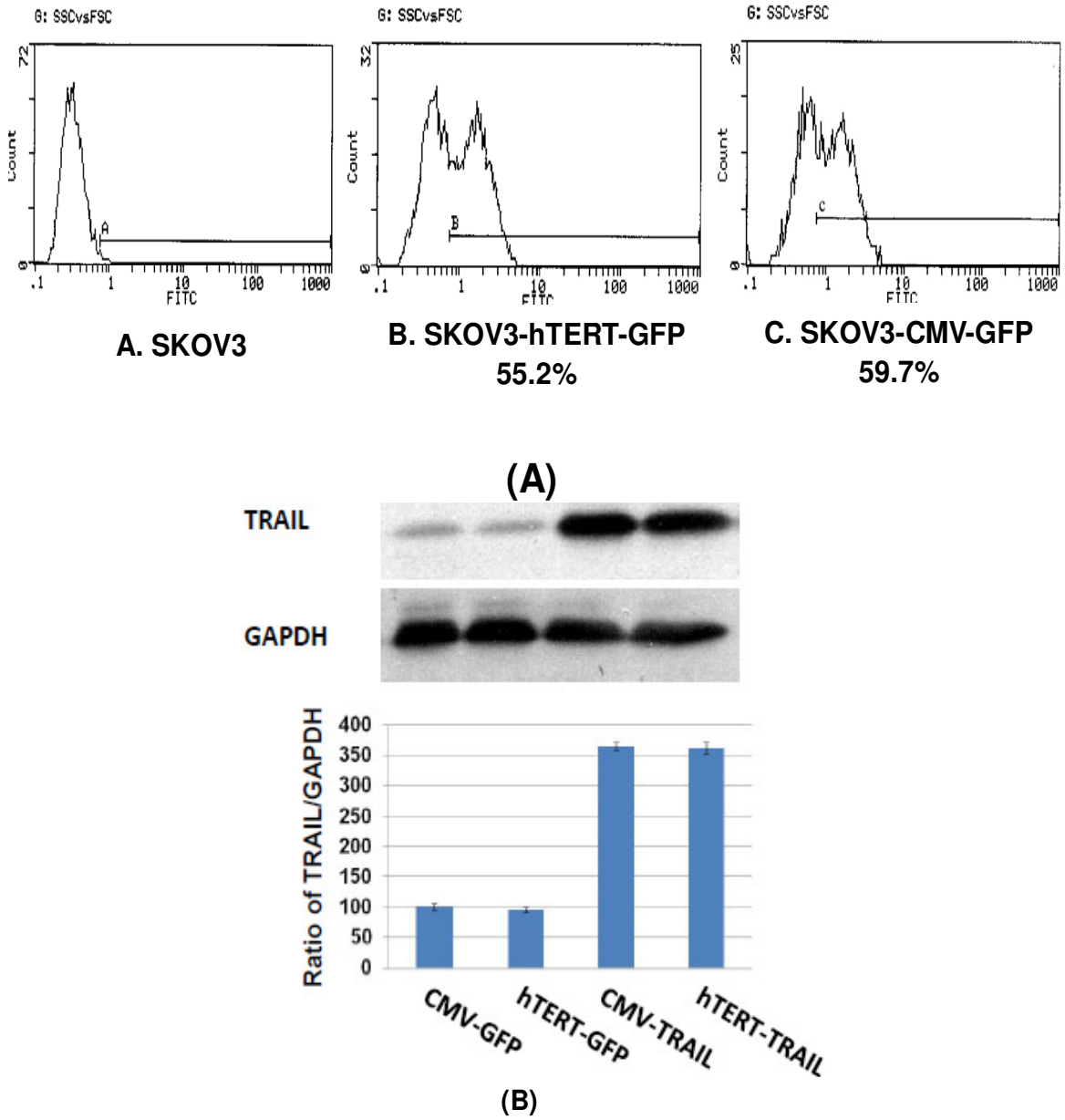

Figure 2. Gene expression efficiency in SKOV3 cancer cells driven by hTERT promoter and CMV promoter. (A) 48 h after transfection, GFP expression in SKOV3 cells was measured by flow cytometry. As indicated, positive rate for hTERT-GFP expression in SKOV3 cells was 55.2% while CMV-GFP expression was 59.7% ($p > 0.05$). (B) 48 h after transfection, TRAIL protein expressions were measured by western blot. An equal loading was verified using GAPDH antibody. All results are representative of three independent experiments.

reduced, only 30% to that of SKOV3 cells ($p<0.01$).

TRAIL expression under hTERT promoter induced increased apoptosis in ovarian cancer cells

To further examine whether the cell growth inhibition was caused through cell apoptosis, we analyzed the apoptotic effect using TUNEL assay. Our data (Figure 3B) indicated that in SKOV3-hTERT-GFP and SKOV3-CMV-GFP cells, apoptosis rate were 10.2 and 11.6%, while in SKOV3-hTERT-TRAIL and SKOV3-CMV-TRAILcells, apoptosis rates increased to 48.7 and 52.5% respectively. These suggested that TRAIL expression

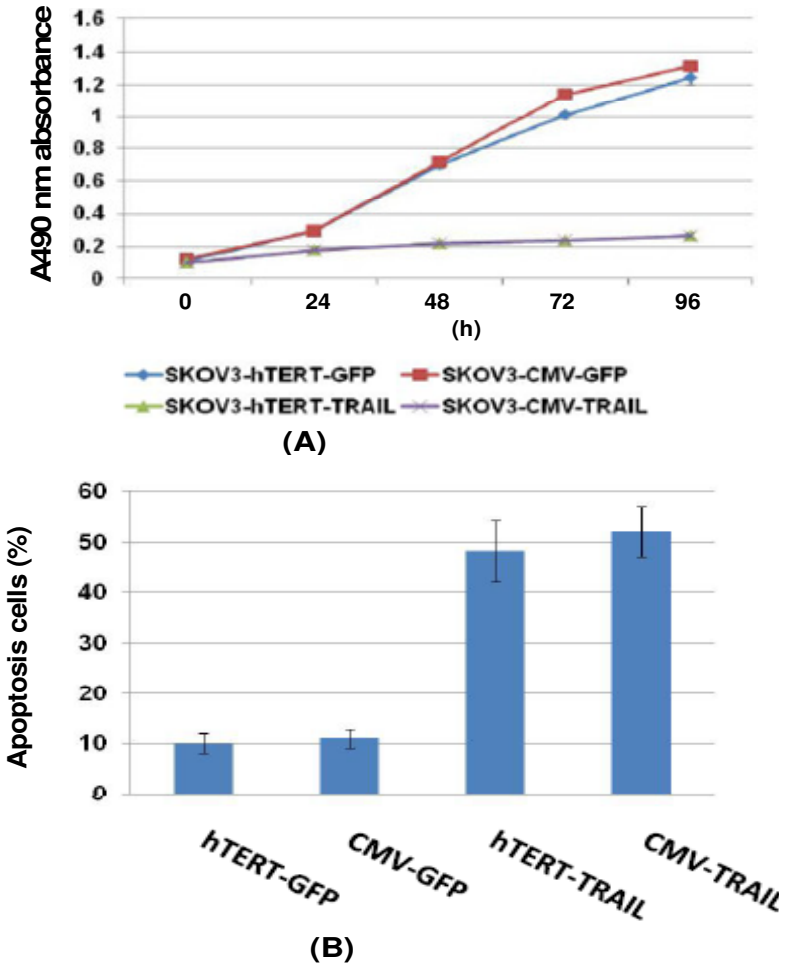

Figure 3. Functional analysis of TRAIL in SKOV3 cells. (A) Cell proliferation assay. SKOV3 cells were transfected with hTERT-GFP, CMV-GFP, hTERT-TRAIL or CMV-TRAIL. After 0, 24, 48, 72 and 96 h, absorbance was measured and recorded. All experiments were performed in triplicate wells and repeated at least three times, and each value represents the mean ± SD of three independent samples. (B). Effect of TRAIL on apoptosis induction. hTERT-GFP, CMV-GFP, hTERT-TRAIL or CMV-TRAIL was transiently cotransfected with pEGFP plasmid into SKOV3 cells. Cells were fixed 48 h after transfection and stained using TUNEL technique. The data shown were the mean percentage ± SD of apoptotic cells among total 100 GFP positive cells counted. The data were collected from at least three independent experiments.

driven by hTERT promoter or CMV promoter could effectively potentiated cell apoptosis in ovarian cancer SKOV3 cells.

TRAIL expression under hTERT promoter has significant antitumor effect against ovarian cancer in human xenograft mouse model

Using a series of *in vitro* assays, we demonstrated that hTERT promoter was comparable in potency to the CMV promoter as controlling the expression of TRAIL gene in SKOV3 cancer cells. We next assessed the functional efficacy of TRAIL *in vivo* using human xenograft mouse model. The tumor size generally reached 3 to 4 mm in diameter after subcutaneously implanted SKOV3 cells in nude mice for 10 days. We then performed intratumoral lipofectamine-mediated transfection of hTERT-TRAIL, CMV-TRAIL, hTERT-EGFP and CMV-EGFP constructs.

Table 1. Colony-formation rate in soft agar assay.

Group	Colony formation rate
SKOV3-hTERT-GFP	100 ± 4.5
SKOV3-CMV-GFP	95 ± 3.1
SKOV3-hTERT-TRAIL	35 ± 3.3*
SKOV3-CMV-TRAIL	36 ± 3.2#

*p<0.01, compared with the groups SKOV3-hTERT-GFP and SKOV3-hTERT-TRAIL.# p<0.01, compared with the groups SKOV3-CMV-GFP and SKOV3-CMV-TRAIL.

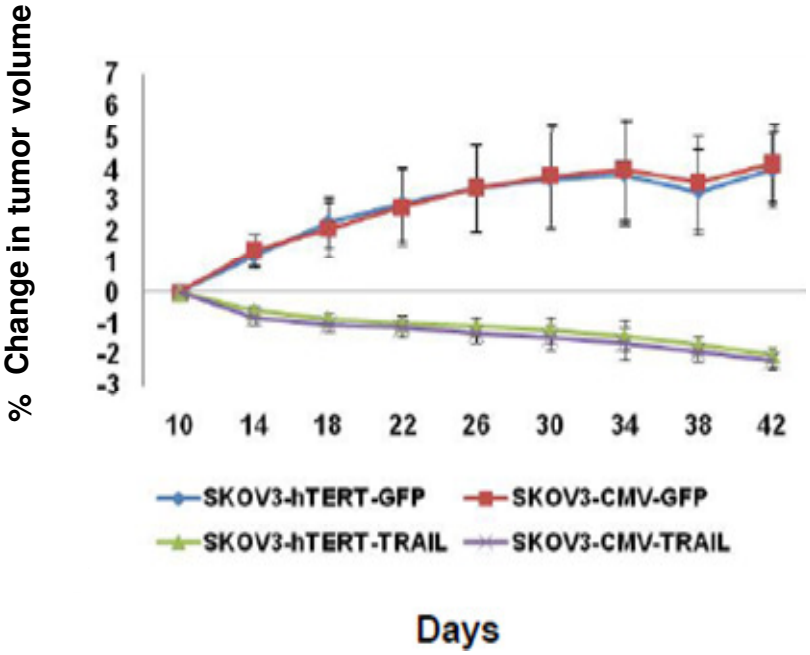

Figure 4. TRAIL expression resulted tumor regression in orthotopic xenograft animal model. Ten days after inoculation of SKOV3 cells subcutaneously in nude mice, hTERT-TRAIL, CMV-TRAIL, hTERT-EGFP and CMV-EGFP constructs was transfected intratumorally three times at day 10, 14 and 18. Ten mice were used in each treatment group. Tumor volume was measured with calipers every 4 days for 6 weeks and calculated as lengthXwidth^2/2. Each data point represents the mean ± SD of the percentage of change in tumor volume. Significant tumor regression was observed in all mice that received TRAIL gene transfection compared to the groups of mice receiving GFP gene transfection (p<0.01).

As shown in Figure 4 for growth curves of implanted tumors, 30 days after intratumor transfection, the two control groups without TRAIL gene transfer showed a continuous increase in tumor size, while the other two groups with TRAIL gene transfer all showed suppression of tumor growth (p<0.01). In addition, hTERT-TRAIL led to a substantial suppression of tumor growth which was comparable to that of CMV-TRAIL (P = 0.1).

DISCUSSION

Though ovarian cancer accounts for only 4% of cancers in women, it is the leading cause of death from gynaecological malignancies all over the world. Since ovarian cancer tends to be asymptomatic until it is well advanced, it is difficult to diagnosis at early stages. Despite progress in surgical and chemotherapeutic

therapies, sufficient results for the treatment of advanced cancer have not been achieved.

One of the ongoing but less developed areas of cancer treatment is gene therapy, which has the potential to significantly improve therapeutic outcomes (Wu, 2009; Lopez-Beltran et al., 2007). Targeting the apoptotic machinery of malignant cells is an attractive concept to combat cancer, which is currently exploited for the proapoptotic members of the TNF ligand family at various stages of preclinical and clinical development (Croft, 2009; Bertazza and Mocellin, 2008; Van Horssen et al., 2006. TRAIL has been under intense focus because of its remarkable ability to induce apoptosis in malignant human cells while leaving normal cells unscathed.

Consequently, activation of the apoptotic signaling pathway from the death-inducing TRAIL receptors provides an attractive, biologically targeted approach to cancer gene therapy. TRAIL-based approaches to cancer therapy vary from systemic administration of recombinant, soluble TRAIL protein with or without the combination of traditional chemotherapy, radiation or novel anti-cancer agents to agonistic monoclonal antibodies directed against functional TRAIL receptors to TRAIL gene transfer therapy (Holoch and Griffith, 2009). Though successful as it is, a major concern facing the routine use TRAIL in the clinic is to achieve tumor-specific treatment.

Telomerase has become an interesting and important target for cancer therapeutics because positive telomerase expression, which allows tumours to maintain their telomeres leading to long-term survival, is present in most cancer types (Proctor et al., 2009; Kyo et al., 2008). Importantly, the fact that telomerase is generally undetectable in normal tissues is because it is under tight control of hTERT promoter which is specifically active in tumour tissues (Braunstein et al., 2001). Therefore, the feasibility of using the hTERT promoter to restrict the expression of therapeutic transgenes to tumours would be expected.

To investigate the possibility of the utilization of the hTERT promoter in targeted gene therapy, we constructed eukaryotic expression vector expressing TRAIL controlled by the hTERT promoter for the induction of specific cancer cell death. Generally, mammalian promoters are characterized by low-expression activity compared with viral regulatory elements, resulting in inefficient activation of transcription and, consequently, low levels of production of therapeutic protein. In the current study, therefore, we initially compared the activity of hTERT promoter with CMV promoter in vitro. We showed that the hTERT promoter could drive gene expression specifically in cancer cells with comparable activity to that of CMV promoter. This is consistent with what was previously reported for TNF-a expression in melanoma (Xiang et al., 2009) and NAT (noradrenaline transporter) expression in glioma (Mairs et al., 2007).

We then investigated the functional efficacy of TRAIL expression driven by hTERT promoter and CMV promoter both in vitro and in vivo. We demonstrated evidence that TRAIL expression driven by hTERT promoter can specifically locate cancer cells, but not normal epithelial cells. In ovarian cancer SKOV3 cell, the hTERT promoter activity is comparable to the CMV promoter activity. TRAIL expression driven by hTERT promoter or CMV promoter can effectively inhibit cancer cell growth, induce apoptosis *in vitro* and retard cancer growth *in vivo*. Our results on the strength and tumor selectivity of the hTERT promoter are consistent with those from earlier studies. For example, it has been reported that the induction of the Bax (Gu et al., 2000), caspase-6 gene (Komata et al., 2001), cytosine deaminase (Wang, et al., 2003) or thymidine kinase gene (Majumdar et al., 2001) via the hTERT promoter elicited tumor-specific apoptosis in vitro, suppressed tumor growth in nude mice, and prevented the toxicity against normal organs. These findings, including the present study, strongly suggest that hTERT promoter is clearly a strong and tumor-specific promoter with high potential in targeted cancer gene therapy.

In our *in vivo* study in athymic mice, we assessed the effects of TRAIL gene under the control of the hTERT promoter on xenograft growth through intratumoral injection. It is not known, if systemic administration would also achieve high specificity. This is our ongoing work. Moreover, we are in the process of examining the effects of TRAIL expression driven by hTERT promoter in combination with other chemotherapy drugs.

In summary, experimental results derived from multiple sets of experimentations in this paper supported our initial hypothesis that hTERT promoter could efficiently regulate the expression of TRAIL, thus specifically induce apoptosis of cancer cells.

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