

Construction of a novel vector expressing Survivin-shRNA and fusion suicide gene yCDglyTK and its application in inhibiting proliferation and migration of colon cancer cells

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Abstract. Despite progress achieved in cancer chemotherapy in recent decades, adverse effects remain a limiting factor for a number of patients with colorectal cancer, suggesting the requirement for novel therapeutic strategies. Gene therapy appears to be a promising strategy for treating cancer. The present study aimed to investigate the anti-tumor effect of a combined gene therapy, using Survivin downregulation by RNAi and a fusion suicide gene yCDglyTK therapy system. A triple-gene vector expressing Survivin-targeted small hairpin RNA (Survivin-shRNA) and fusion suicide gene yCDglyTK was constructed, and administered to HCT116 cells. Survivin expression decreased significantly and yCDglyTK fusion gene expression was confirmed by both reverse transcription-quantitative polymerase chain reaction and western blot analysis. Introduction of Survivin-shRNA into yCDglyTK/prodrug system eradicated colon cancer cells and induced apoptosis more effectively. Furthermore, this therapeutic system is able to inhibit the migration of HCT116 cells. These results indicate that the recombinant plasmid may serve as a novel gene therapy approach to treat colorectal carcinoma.

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide (1). Of patients with CRC, 50-70% are diagnosed at advanced stages (2), and adjuvant chemotherapies are recommended in addition to radical surgery to decrease the possibility of recurrence and increase the success rate. However, adjuvant chemotherapies, which are administrated systemically, are

unable to selectively target cancerous cells and, in turn cause substantial toxicity (3), resulting in an impaired quality of life for patients. Therefore, novel therapeutic strategies are required.

Gene-direct enzyme/prodrug therapy (GEPT), also named suicide gene therapy, has received considerable attention due to its powerful anti-tumor efficacy without side effects (4,5). GEPT is based on the intracellular delivery of genes encoding enzymes that convert nontoxic prodrugs into highly cytotoxic metabolites (6). Well-characterized GEPTs include the herpes simplex virus thymidine kinase/ganciclovir (HSV-TK/GCV) and cytosine deaminase/5-fluorocytosine (CD/5-FC) (7). TK activates GCV to its cytotoxic triphosphate derivative, which inhibits cellular DNA synthesis, whereas CD deaminates 5-FC into the highly toxic 5-fluorouracil (5-FU), which may interfere with nucleoside metabolism and lead to targeted cell death (8). However, GEPT is thought to be insufficient to cure cancer alone (9). Previously, a number of studies have aimed to enhance the therapeutic effect of GEPT through combination with other gene therapies, including immuno-gene (10), anti-oncogene (11) and inhibition of multiple drug resistance gene based on RNAi (12).

Survivin, which is known to be a member of the inhibitor of apoptosis protein family (13), is overexpressed in a number of human cancer types, including CRC (14-16). Recent studies have indicated that Survivin serves an essential role in tumor growth, infiltration and metastasis, and that it is closely associated with the chemo-resistance of cancer cells (17,18). Survivin has become a focus in cancer therapy. RNA interference (RNAi) technology, based on sequence-specific interactions between small interfering RNA (siRNA) and mRNA (19), is post-transcriptional gene silencing. Inhibition of Survivin by RNAi has been demonstrated to restrain tumor growth and metastasis, and increase sensitivity to anti-tumor agents (20). The anti-tumor effect of GEPT is mediated by cytotoxic metabolites of prodrugs, such as 5-FU. The down-regulation of Survivin may help maintain the sensitivity of colorectal cancer cells to the cytotoxic drugs. Therefore, a combination of Survivin-targeted RNAi and the suicide gene may exhibit synergistic effects for cancer treatment.

In the present study, a triple-gene vector expressing Survivin-shRNA and fusion suicide gene yCDglyTK was

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constructed to assess the feasibility of a novel therapeutic vector system involving a combination of GEPT with Survivin-targeted RNAi therapy. This novel vector was delivered into HCT116 cells (a colon cancer cell line) by calcium phosphate nanoparticles (CPNPs), and the anti-tumor effect was studied *in vitro*.

Materials and methods

Reagents. Restriction enzymes *Bsa*I, *Mlu*I, *Xho*I and *Nhe*I were purchased from MBI Fermentas (Thermo Fisher Scientific, Inc., Waltham, MA, USA). T4-DNA ligase (New England Biolabs, Inc., Ipswich, MA, USA), rTaq DNA polymerase (Takara Biotechnology Co., Ltd., Dalian, China), DNA Marker IV, DNA Marker DL2000 (YRbio; Changsha, China), pYr1.1 vector (YRbio) and pUC57 (YRbio) were applied. Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Inc.), MinElute Gel Extraction Kit (Qiagen GmbH; Hilden, Germany), Geneticin (G418; Thermo Fisher Scientific, Inc.), TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), ReverTra Ace reverse transcription kit (Toyobo Co., Ltd., Osaka, Japan), 2X Taq PCR MasterMix (Tiangen Biotech Co., Ltd., Beijing, China), rabbit anti-Survivin antibody (ab76424; 1:5,000; Abcam, Cambridge, UK), mouse anti-TK antibody (sc-53331; 1:200; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), mouse anti- β -actin antibody (A5316; 1:5,000; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), goat anti-rabbit secondary antibody (SA00001-2; 1:2,000; Proteintech Group, Inc., Chicago, IL, USA), goat anti-mouse secondary antibody (SA00001-1; 1:2,000; Proteintech Group, Inc.), rabbit anti-CD antibody (10348-924; 1:200; VWR International; Randor, PA, USA), FITC-Goat Anti-Rabbit antibody (SA00003-2; 1:100; Proteintech Group, Inc.), GCV (Sigma-Aldrich; Merck KGaA), 5-FC (Sigma-Aldrich; Merck KGaA), MTT solution (Sigma-Aldrich; Merck KGaA), dimethyl sulfoxide (Promega Corporation) and pyridine iodide (PI; Sigma-Aldrich; Merck KGaA) were also used in the study.

Construction of Survivin-shRNA expressing plasmid. The Survivin mRNA sequence in GenBank (<https://www.ncbi.nlm.nih.gov/gene>) was searched, and three Survivin-specific target sequences were selected according to the RNAi design tool (https://sg.idtdna.com/site/order/design-tool/index/DSIRNA_CUSTOM). The first siRNA sequence targeted the coding region 118-138 (5'-GAGGCTGGCTTCATCCACTGC-3'), the second sequence targeted the coding region 323-342 (5'-GAGCCAAGAACAATAATGTC-3') and the third sequence targeted the coding region 387-405 (5'-GAAAGTGCGCCGTGCCAT-3'). Oligonucleotides that encoded the corresponding small hairpin RNA (shRNA) were synthesized commercially (Yrbio, Changsha, China), and the sequences are presented in Table I.

The oligonucleotides were annealed in annealing buffer (10 mM Tris-HCl pH 8.0, 50 mM NaCl and 1 mM EDTA), and pYr1.1 vector was digested with *Bsa*I at 37°C overnight. Subsequently, the linear fragment of pYr1.1 and the annealing products were connected at 4°C overnight to construct pYr1.1-Survivin-sh1, pYr1.1-Survivin-sh2 and pYr1.1-Survivin-sh3, respectively. The expression of shRNA

was regulated by the U6 promoter. Then the three interfering plasmids were sequenced. The three interfering plasmids were then transfected into HCT116 cells using Lipofectamine 2000 according to the manufacturer's instructions, and the protein expression of Survivin was evaluated by western blot analysis, as described below. pYr1.1-Survivin-sh2 was confirmed to be the most effective interfering plasmid.

Construction of the triple-gene plasmid. The suicide gene should be expressed only in cancer cells, and the human telomerase reverse transcriptase promoter (hTERTp) was used to observe target expression. The hTERTp was synthesized by Yrbio, according to a previous study (21), and the sequence was: 5'-ACGCGTGCTCCCAGTGGATTCGCGGGCACA GACGCCAGGACCGCGCTCCCCACGTGGCGGAG GGACTGGGGACCCGGGCACCCGTCCTGCCCTTCAC CTTCCAGCTCCGCCTCCTCCGCGCGGACCCCGCCCC GTCCCGACCCCTCCCGGGTCCCGGGCCAGCCCCCT CCGGGCCCTCCCAGCCCCCTCCCCTTCCTTTCCGCGG CCCC GCCCTCTCCTCGCGGCGCGAGTTTCAGGCAGC GCTGCGTCTGCTGCGCACGTGGGAAGCCCTGG CCCC GGCCACCCCGCGGGCTAGC-3' (the underlined sections were *Mlu*I and *Nhe*I restriction sites, respectively), and was subcloned into pUC57 vector, which was named pUC57-hTERTp. pUC57-hTERTp and pYr1.1 were digested by *Mlu*I and *Nhe*I at 37°C overnight, respectively, and the linear fragments were connected by T4 DNA ligase at 4°C overnight to construct pYr1.1-hTERTp. A plasmid carrying fusion suicide gene yCDglyTK was constructed as described in our previous study (22), which was stored in the department of Gastroenterology, Xiangya Hospital of Central South University (Changsha, China). The fusion suicide gene yCDglyTK was amplified through polymerase chain reaction (PCR). Primer sequences used were as follows: P1, 5'-CTA GCTAGCGCCACCATGGTGACAGGGGAATGGCAA-3' (*Nhe*I restriction site was introduced), and P2, 5'-CCGCTC GAGTCAGTTAGCCTCCCCATCT-3' (*Xho*I restriction site was introduced). The reaction mixture for PCR contained the following: 0.25 μ l P1 (10 μ M), 0.25 μ l P2 (10 μ M), 19.75 μ l dH₂O, 2.5 μ l 10X LA PCR buffer (Mg²⁺ Plus), 1 μ l dNTPs (2.5 mM), 0.25 μ l LA Taq polymerase and 1 μ l template. The thermal cycle profile for PCR was 94°C for 5 min, followed by 30 cycles of 20 sec at 94°C, 25 sec at an annealing temperature of 58°C, 105 sec at 72°C, and an additional 3 min incubation at 72°C following completion of the last cycle for extension. Following electrophoresis on 1% agarose gel, PCR products were extracted and stored at 4°C.

PCR products of yCDglyTK and pYr1.1-hTERTp were subsequently digested by *Nhe*I and *Xho*I respectively at 37°C overnight, and the two linear fragments were connected at 4°C overnight to develop the plasmid pYr1.1-hTERTp-yCDglyTK. In this process, the enhanced green fluorescent protein (EGFP) of pYr1.1-hTERTp was replaced by yCDglyTK.

pUC57-hTERTp and pYr1.1-Survivin-sh2 were digested by *Mlu*I and *Nhe*I respectively at 37°C overnight, and the linear fragments were connected at 4°C overnight to construct pYr1.1-hTERTp-Survivin-sh2. Subsequently, PCR products of yCDglyTK and pYr1.1-hTERTp-Survivin-sh2 were digested by *Nhe*I and *Xho*I respectively at 37°C overnight, and the two linear fragments were connected (at 4°C overnight) to construct

Table I. Sequences of oligonucleotides encoding Survivin-shRNA.

Survivin-shRNA	Sequences of oligonucleotides
Survivin-sh1	Forward: 5'-CACCGAGGCTGGCTTCATCCACTGCCTCGAGGCAGTGGATGAAGCCAGCCTCTTTTTTTG-3' Reverse: 5'-AGCTCAAAAAAGAGGCTGGCTTCATCCACTGCCTCGAGGCAGTGGATGAAGCCAGCCTC-3'
Survivin-sh2	Forward: 5'-CACCGAGCCAAGAACAAAATTGCTTCAAGAGAGCAATTTTGTCTTGGCTCTTTTTTTG-3' Reverse: 5'-AGCTCAAAAAAGAGCCAAGAACAAAATTGCTCTCTTGAAGCAATTTTGTCTTGGCTC-3'
Survivin-sh3	Forward: 5'-CACCGAAAGTGCGCCGTGCCATCTTCAAGAGAGATGGCACGGCGCACTTTCTTTTTTTG-3' Reverse: 5'-AGCTCAAAAAAGAAAGTGCGCCGTGCCATCTCTCTTGAAGATGGCACGGCGCACTTTC-3'

Table II. Plasmids used in the present study.

Plasmids	Abbreviations	Promoters	Inserts
pYr1.1	pYr1.1	hU6	EGFP
pYr1.1-Survivin-sh1/2/3	shSur1/2/3	hU6	Survivin-shRNA1/2/3
pYr1.1-hTERTp	pYr1.1-hTERTp	hTERTp and hU6	EGFP
pYr1.1-hTERTp-yCDglyTK	hTERTp-CDTK	hTERTp and hU6	yCDglyTK
pYr1.1-hTERTp-yCDglyTK-sh Survivin2	CDTK-shSur	hTERTp and hU6	yCDglyTK and Survivin-shRNA2

EGFP, enhanced green fluorescent protein; hTERTp, the human telomerase reverse transcriptase promoter; CDTK, fusion suicide gene involving yeast CD gene and HSV-TK gene; shSur, Survivin-targeted small hairpin RNA.

a novel triple-gene vector pYr1.1-hTERTp-yCDglyTK-shSurvivin2. Plasmids used in the current study are presented in Table II.

Cell line and cell culture. HCT116 (a human colon cancer cell line) and human fibroblasts obtained from the Central Laboratory of the Second Xiangya Hospital, Central South University (Changsha, China), were used in the present study. The present study was approved by the ethics committee of the Second Xiangya Hospital, Central South University (Changsha, China) and informed consent was obtained from patients prior to the use of human tissue. Cells were cultured in RPMI 1640 medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS, Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) and maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Analysis of EGFP expression. HCT116 and human fibroblasts were seeded in 6-well plates at a density of 2x10⁵ cells/well. As described in a previous study (22), calcium phosphate nanoparticles (CPNPs) were produced, and 2 µg DNA (pYr1.1-hTERTp) was mixed with 20 µg CPNPs to form the CPNP-DNA complex, which was then added to each well. The expression of EGFP was analyzed 48 h later using a fluorescence microscope (DMI 4000B; Leica Microsystems GmbH, Wetzlar, Germany).

Stable transfection in vitro. HCT116 cells were seeded in 6-well plates at a density of 2x10⁵ cells per well. When the cell monolayer reached 70-80% confluence, hTERTp-CDTK and CDTK-shSur were mixed with CPNPs respectively. Each of the CPNP-DNA complexes was added to different 6-well plates as described previously (22). The next day, a 1:10 passage of the transfected HCT116 cells was performed, followed by the addition of 400 µg/ml G418 for selection. G418-resistant clones were isolated and expanded in RPMI-1640 culture medium containing 200 µg/ml G418. Surviving colonies transfected with hTERTp-CDTK or CDTK-shSur were renamed HCT/CDTK, or HCT/CDTK-shSur, respectively, and subjected to further studies.

Reverse transcription-PCR (RT-PCR). Total RNA from parental and transfected HCT116 cells was extracted using TRIzol reagent. The quantity and quality of RNA were assessed by absorbance at 260 nm and 280 nm using an ultraviolet spectrophotometer (DU800; Beckman Coulter, Inc., Brea, CA, USA). The RT reaction was performed using the ReverTra Ace reverse transcription kit according to the manufacturer's protocol. Subsequently, PCR was performed on the cDNA product. For yCDglyTK, a PCR product of 707 bp was produced by forward primer 5'-GGGAGATTAGAGGGCAAAGTGT-3' and reverse primer 5'-ACGGCGTCGGTCACGGCA TAA-3'. For Survivin, a PCR product of 107 bp was produced

by forward primer 5'-CATCCTGCGTCTGGACCTGG-3' and reverse primer 5'-TAATGTACACGACGATTTC-3'. β -actin was used as an internal control, and the forward primer was 5'-AGCGAGCATCCCCAAAGTT-3' and the reverse primer was 5'-GGGCACGAAGGCTCATCATT-3'. The thermal cycle profile for PCR was 94°C for 3 min, followed by 28 cycles of 30 sec at 94°C, 30 sec at an annealing temperature of 55°C and 60 sec at 72°C. PCR products were electrophoresed on 2% agarose gels, and visualized using gel image analysis system (BIO-PRO, SIM International group Co., Ltd., Los Angeles, CA, USA) and analyzed by BandsScan 5.0 (<http://www.bbioso.com/download/58-140-1.html>).

Western blot analysis. Parental and transfected HCT116 cells were lysed in radioimmunoprecipitation assay buffer on ice containing phenylmethylsulfonyl fluoride for 30 min with occasional agitation. The lysates were transferred to E-tubes and clarified by centrifugation at 14,000 $\times$ g for 15 min at 4°C. The supernatant was collected and protein concentrations were evaluated using a BCA protein assay. Identical amounts (40 μ g protein) of cell lysates were separated via 15% SDS-PAGE and transferred to polyvinylidene fluoride membranes (GE Healthcare, Chicago, IL, USA). The membranes were incubated in blocking solution, consisting of 5% skim milk in Tris buffered saline with Tween-20 [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween-20], for 1 h at room temperature, then probed with rabbit anti-Survivin antibody, rabbit anti-TK antibody or mouse anti- β -actin antibody at 4°C overnight. This was followed by incubation with their respective peroxidase-conjugated secondary antibodies for 1.5 h at room temperature. The blots were visualized by the enhanced chemiluminescence detection system (GE Healthcare) and analyzed by BandsScan 5.0.

Immunofluorescence assay. Parental and transfected HCT116 cells (2 $\times$ 10⁵ cells per well) were fixed in 4% formaldehyde for 20 min at room temperature. Cells were washed with cold PBS three times, then permeabilized in 0.3% Triton X-100 and blocked with 1% bovine serum albumin for 30 min at room temperature. The cells were then treated with rabbit anti-CD antibody overnight at 4°C in a humidified chamber. Then, cells were incubated with anti-rabbit immunoglobulin G-fluorescein isothiocyanate antibody for 1 h at 37°C in the dark. After being washed three times with PBS, coverslips were mounted with a drop of mounting medium (Beyotime Institute of Biotechnology, Shanghai, China), sealed with clear nail polish and visualized using a fluorescence microscope (magnification, $\times$ 200).

MTT assay. HCT116 cells (transfected and untransfected) were seeded in 96-well plates at a density of 6,000 cells per well. The next day (at 37°C), cells were treated with prodrugs: 200 μ g/ml 5-FC and 16 μ g/ml GCV, which have been confirmed to have limited toxicity on untransfected cells in a previous study (22), and cell viability was measured after 24, 48, 72 and 96 h of incubation at 37°C. A volume of 20 μ l MTT solution (5 mg/ml) was added and cells were further incubated at 37°C for 4 h. Then, the culture medium was removed and replaced with 200 μ l dimethyl sulfoxide to dissolve the blue crystals. The optical density (OD) was determined using a multi-well plate

reader (Stat-Fax-2100; Awareness Technologies, Westport, CT, USA) by measuring absorbance at 570 nm (OD570), with the absorbance at 690 nm as a reference. The background absorbance of medium was also subtracted. Cell growth curves were produced with culture time on the horizontal axis and OD570 on the vertical axis.

Cell apoptosis analyzed by flow cytometry. A flow cytometry assay was performed to evaluate the loss of cell viability in each experimental group. Parental and transfected HCT116 cells were seeded into 75 cm² cell culture flasks at a density of 2 $\times$ 10⁶ cells per flask. RPMI 1640 medium (supplemented with 10% FBS) with 200 μ g/ml 5-FC and 16 μ g/ml GCV was added when the cells reached 70% confluence. 48 h later, the cells were pelleted by centrifugation at 800 $\times$ g, washed with cold PBS twice, fixed in 75% ethanol for 30 min at 4°C and resuspended in a staining solution of PI (50 mg/ml) for 30 min at 37°C. Finally, the cell apoptosis rate was analyzed using flow cytometry (FACSCanto, BD Biosciences; San Jose, CA, USA).

Migration assay. A wound healing assay was applied to analyze cell migration. HCT116 cells (transfected and untransfected) were seeded in 6-well plates at a density of 5 $\times$ 10⁵ cells/well in RPMI 1640 medium with 10% FBS for 24 h at 37°C to reach 95% confluence. The monolayers were then scratched with a 200 μ l pipette tip. The cells were washed three times with PBS, and cultured in RPMI 1640 medium without FBS for 24 h at 37°C. Migration of the cells was detected under a light microscope. The wound margin distances between the two edges of the migrating cell sheets were measured at 0 and 24 h following scratching. The relative migrating distance of cells was measured as follows: Distance of cell migration/the distance measured at 0 h.

Statistical analysis. All results were expressed as mean $\pm$ standard deviation. Statistical analysis was performed using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). The Student's t-test and one-way analysis of variance assessments followed by the Student-Newman-Keuls test was performed. $P < 0.05$ was considered to indicate statistically significant differences.

Results

Construction of the plasmid pYr1.1-hTERTp-yCDglyTK-shSurvivin2. Three interfering plasmids targeting Survivin were constructed and the most effective plasmid, pYr1.1-Survivin-sh2, was selected. Subsequently, hTERTp was cloned into pYr1.1 to obtain pYr1.1-hTERTp, and the specificity of hTERTp was confirmed by fluorescence microscopy, as presented in Fig. 1A. Then, yCDglyTK was cloned into pYr1.1-hTERTp to generate pYr1.1-hTERTp-yCDglyTK. Finally, Survivin-shRNA from pYr1.1-Survivin-sh2 was cloned into pYr1.1-hTERTp-yCDglyTK to develop the triple-gene plasmid pYr1.1-hTERTp-yCDglyTK-shSurvivin2. In this novel triple-expressing plasmid, the Survivin-shRNA sequence was driven by a U6 promoter, whereas fusion suicide gene yCDglyTK was regulated by hTERTp. The construction scheme of the triple-gene plasmid

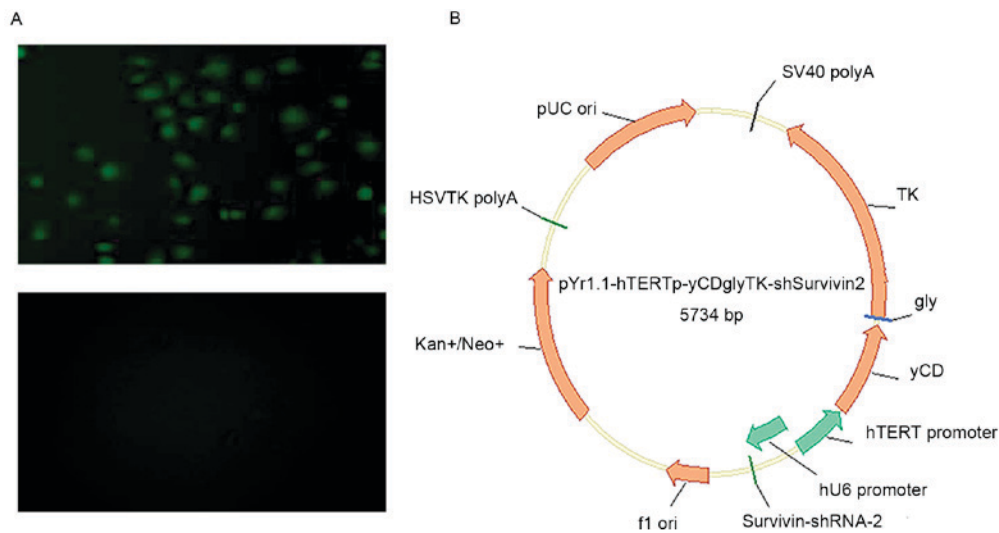


Figure 1. Construction of the pYr1.1-hTERTp-yCDglyTK-shSurvivin2 plasmid. (A) Expression of EGFP in HCT116 cells and human fibroblasts by transfection with pYr1.1-hTERTp. Top image demonstrates the EGFP-positive expression of HCT116 cells by fluorescence microscopy; bottom image demonstrates the negative expression of EGFP in fibroblasts (magnification, x200). (B) Construction scheme of a novel plasmid pYr1.1-hTERTp-yCDglyTK-shSurvivin2. EGFP, enhanced green fluorescent protein; TK, thymidine kinase; yCD, yeast cytosine deaminase; hTERT, human telomerase reverse transcriptase; Survivin-shSur, Survivin-targeted small hairpin RNA.

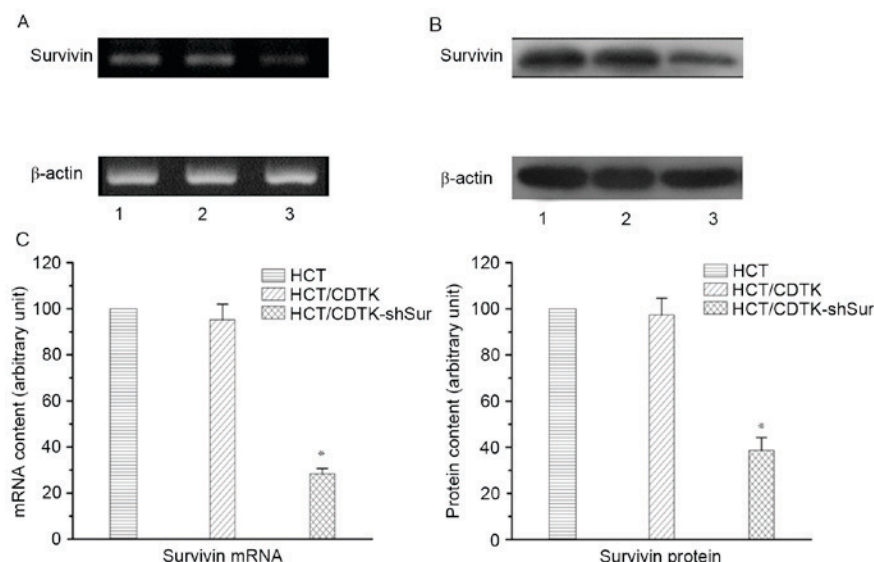


Figure 2. Inhibition of Survivin mRNA and protein expression by transfection with CDTK-shSur. Representative Survivin mRNA and protein expression were analyzed by (A) RT-qPCR and (B) western blot analysis, respectively. β -actin was used as an internal control. Lane 1, parent HCT116; lane 2, HCT/CDTK; lane 3, HCT/CDTK-shSur. (C) Density of each band was measured, densities of Survivin were normalized against corresponding β -actin signals, and relative intensities were expressed in arbitrary units where the intensity of parent HCT116 cells was set to 100%. * $P < 0.01$ vs. all other groups. The data are expressed as mean + standard deviation from three independent experiments. CDTK, fusion suicide gene involving yeast CD gene and herpes simplex virus thymidine kinase gene; shSur, Survivin-targeted small hairpin RNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

pYr1.1-hTERTp-yCDglyTK-shSurvivin2 is presented in Fig. 1B.

Establishment of stably transfected cell lines. hTERTp-CDTK and CDTK-shSur were administered to HCT116 cells using CPNPs. Following G418 selection, stably transfected cell lines were established. HCT116 cells transfected with hTERTp-CDTK were named HCT/CDTK, and those transfected with CDTK-shSur were named HCT/CDTK-shSur. RT-qPCR and western blot analysis were performed to determine the expression of Survivin and yCDglyTK, and immunofluorescence was

conducted to determine the expression of yCDglyTK (Figs. 2 and 3, respectively). Compared with parent HCT116 cells and HCT/CDTK, mRNA and protein levels of Survivin were significantly decreased in HCT/CDTK-shSur ($P < 0.01$; Fig. 2C). yCDglyTK was revealed to only be expressed in HCT/CDTK and HCT/CDTK-shSur cells (Fig. 3).

CDTK-shSur/prodrug system induced cytotoxicity. Following 48 h treatment with 5-FC and GCV, the OD570 of parental HCT116 cells was markedly increased compared with HCT/CDTK and HCT/CDTK-shSur cells (Fig. 4A). Over time,

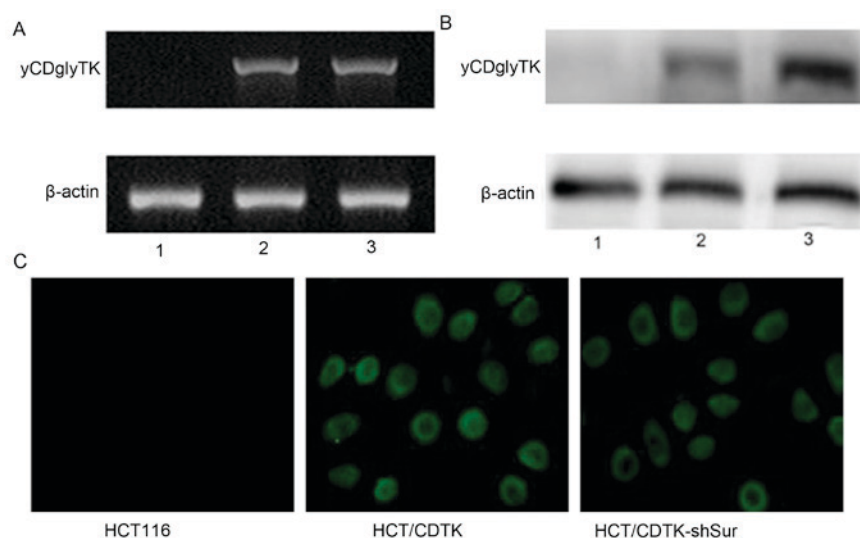


Figure 3. Expression of yCDglyTK by transfection with CDTK-shSur. Representative yCDglyTK mRNA and protein expression were analyzed by (A) RT-qPCR and (B) western blot analysis, respectively. β-actin was used as an internal control. Lane 1, parent HCT116; lane 2, HCT/CDTK; lane 3, HCT/CDTK-shSur. (C) Representative yCDglyTK protein expression detected by immunofluorescence assays. Magnification, x200. CDTK or yCDglyTK, fusion suicide gene involving yeast CD gene and herpes simplex virus thymidine kinase gene; shSur, Survivin-targeted small hairpin RNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

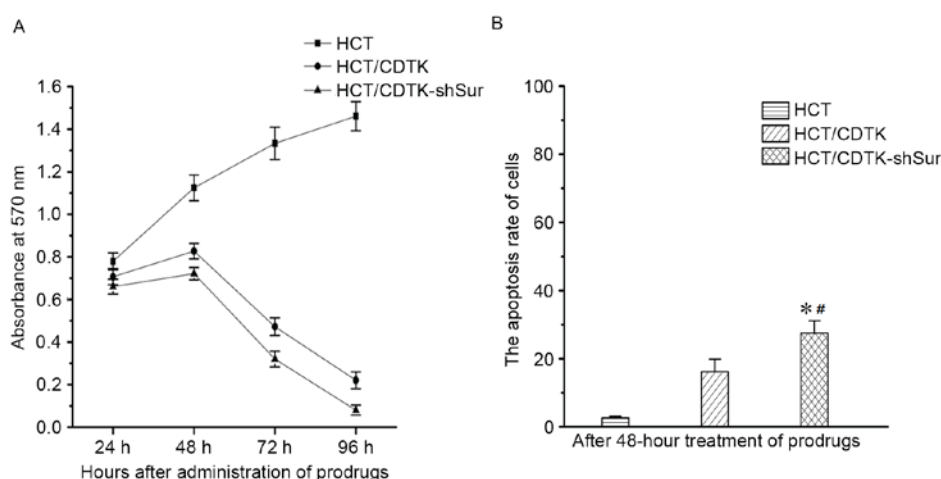


Figure 4. CDTK-shSur/prodrug system induces cytotoxicity and apoptosis. (A) Growth curves of HCT116 cells (transfected and untransfected) following administration of 5-FC and GCV. HCT116 cells (transfected and untransfected) maintained in culture medium containing 5-FC (200 μg/ml) and GCV (16 μg/ml). At 24, 48, 72 and 96 h, cells of each group were subjected to MTT assays. Cell growth curves were plotted with culture time as the horizontal axis and OD570 as the vertical axis. (B) HCT116 cells (transfected and untransfected) were treated with prodrugs (5-FC and GCV) for 48 h, and the apoptosis rate of each group was measured by flow cytometry. * $P < 0.05$ vs. HCT group and # $P < 0.05$ vs. HCT/CDTK group. Data are presented as mean + standard deviation. CDTK, fusion suicide gene involving yeast CD gene and herpes simplex virus thymidine kinase gene; shSur, Survivin-targeted small hairpin RNA; 5-FC, 5-fluorocytosine; GCV, ganciclovir; OD, optical density.

untransfected HCT116 cells sustained a high rate of proliferation, whereas the OD570 of HCT/CDTK and HCT/CDTK-shSur cells decreased markedly, suggesting that the majority of cells were killed. OD570 of HCT/CDTK-shSur remained the lowest throughout.

CDTK-shSur/prodrug system induced cell apoptosis. Each group was treated with prodrugs (5-FC and GCV) for 48 h, and then subjected to flow cytometry to measure the apoptosis rate (Fig. 4B). The percentage of apoptotic cells in untransfected HCT116 cells was $2.63 \pm 0.48\%$, in HCT/CDTK cells was $16.17 \pm 3.71\%$ and in HCT/CDTK-shSur cells was $27.50 \pm 3.62\%$.

The apoptosis rate of HCT/CDTK-shSur cells was significantly higher in comparison with the untransfected HCT116 and HCT/CDTK cells ($P < 0.05$; Fig. 4B), indicating that the CDTK-shSur/prodrug therapy system may induce cell apoptosis more effectively.

CDTK-shSur inhibits cancer cell migration. The migration ability of HCT116 cells was measured using a wound healing assay 24 h following scratching. As presented in Fig. 5, compared with the parental HCT116 cells, the migration of HCT/CDTK-shSur cells decreased significantly ($P < 0.01$; Fig. 5B).

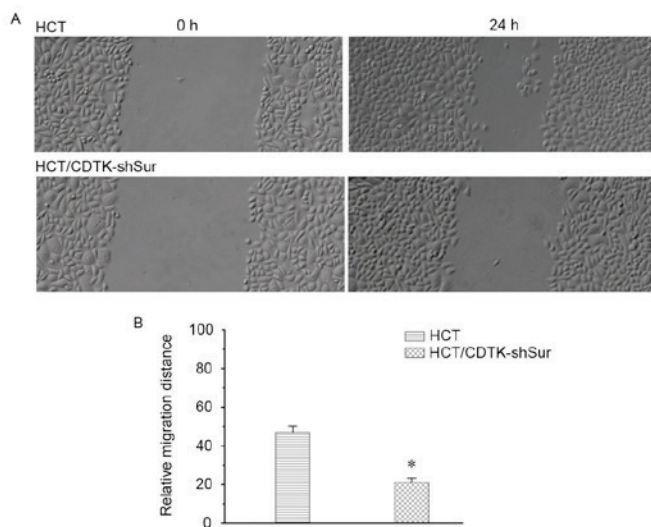


Figure 5. CDTK-shSur inhibits cancer cell migration. (A) Representative images of parent HCT116 and HCT/CDTK-shSur cells in wound healing assay (magnification, $\times 100$). (B) Quantitative analysis of the migration was made by measuring the migration distance 24 h after scratching. * $P < 0.01$ vs. HCT. Data are presented as mean + standard deviation. CDTK, fusion suicide gene involving yeast CD gene and herpes simplex virus thymidine kinase gene; shSur, Survivin-targeted small hairpin RNA.

Discussion

Gene therapy has emerged as a promising strategy for treating malignant tumors (23). As the genesis, development and metastasis of cancer is a complicated process involving multiple factors (24), single gene therapy alone is not effective enough to eradicate cancer cells. Combination gene therapy may be an efficient approach to obtaining greater anti-tumor efficacy. Combination gene therapy may be achieved by co-transferring vectors carrying different genes; however, it is impossible to ensure that all of the different vectors are delivered into the cell simultaneously. The approach of one vector expressing multiple therapeutic genes has been suggested to enhance the therapeutic efficacy (25-27). In the current study, a triple-gene vector expressing Survivin-shRNA and fusion suicide gene yCDglyTK was constructed, in which Survivin-shRNA was regulated by U6 promoter whereas fusion suicide gene yCDglyTK was driven by hTERTp.

Different GEPTs exhibit different characteristics (7). For example, the HSV-TK/GCV system has a more powerful killing efficacy, whereas the CD/5-FC system exerts a superior bystander effect. Furthermore, cell type dependency may exist with GEPT, as HSV-TK/GCV is typically employed in treating gliomas (28), and the CD/5-FC system is often adopted in treating gastrointestinal tumors (29). Double suicide gene combined with HSV-TK/GCV and CD/5-FC may break the dependence of tumor cell types and exhibit a synergistic effect (30). The suicide gene should be expressed only in cancer cells, so GEPT may be regarded as intratumoral chemotherapy and cause little systematic toxicity. In a previous study, a vector expressing the fusion suicide gene yCDglyTK was constructed, and a CEA promoter was used to drive the expression of yCDglyTK, a treatment that specifically killed CEA-positive cancer cells (22). However, not all colorectal cancer cells are CEA-positive (31), and

yCDglyTK driven by a CEA promoter has little effect on the CEA-negative cancer cells. Therefore, in order to expand the applicability of fusion suicide gene therapy, a more prevalent promoter is required. Telomerase is activated in $>85\%$ of all malignant tumor cells, including colorectal cancer cells, but is repressed in normal somatic cells (32-34), the transcriptional activity that is regulated by hTERTp. hTERTp was confirmed to drive specific target gene expression in various tumor cells (9,35-37). Therefore, hTERTp was used in the current study to cause tumor-specific gene expression of yCDglyTK. When pYr1.1-hTERTp was delivered into both HCT116 cells and human fibroblasts, EGFP was only expressed in HCT116 cells and not in human fibroblasts, suggesting that hTERTp was specific enough to drive target gene in cancer cells.

The function of Survivin in tumor progression, metastasis and chemo-resistance has been well documented (38). In the present study, RNAi technology was used to inhibit its expression. Three Survivin-specific target sequences were selected and corresponding Survivin-shRNA expression plasmids were developed, from which the more effective one was selected. Introduction of a Survivin-targeted shRNA increased the cytotoxicity of yCDglyTK. The reasons for this synergistic effect may be as follows: Inhibition of Survivin may promote cell apoptosis and decrease cell mitosis (39); or downregulation of Survivin may maintain and enhance the sensitivity of colorectal cancer cells to cytotoxic metabolites of prodrugs. Furthermore, HCT116 cells transfected with CDTK-shSur exhibited a decreased migration ability, which determines invasiveness and metastasis of cancer cells, even without the presence of prodrugs. These data demonstrated that a combination of Survivin-siRNA and yCDglyTK may be a promising approach to treating cancer in the future.

The novel triple-gene plasmid produced in the current study may eradicate colon cancer cells and decrease their migration effectively *in vitro*. However, there are potential limitations of this novel system. Survivin was revealed to be expressed in normal cells, such as T-cells, hematopoietic progenitor cells, vascular endothelial cells, liver cells, gastrointestinal tract mucosa and polymorphonuclear cells (40), and participates in numerous cell processes including apoptosis, cell proliferation, cell cycle, chromosome movement, mitosis and regulation of response to cellular stress (41). The U6 promoter is not tissue-specific, and CPNPs do not target specific tissues. Strategies aiming to improve the safety of RNAi-based gene therapy are therefore required.

In conclusion, the current study has demonstrated that a combination of Survivin-targeted RNAi and suicide gene therapies exhibits a synergistic effect. Introduction of Survivin-shRNA into the CDTK/prodrug system may be an effective and feasible strategy to eradicate colon cancer cells and inhibit their migration *in vitro*. Although there are a number of limitations to be resolved for further application, the current study provides a novel gene therapy strategy for treating colorectal cancer.

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