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 administered 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 downregulation 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...
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 BsaI, MluI, XhoI and NheI 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 Extration Kit (Qiagen GmbH; Hilden, Germany), Genetecin (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 Biotechnology, Inc., Dallas, TX, USA), mouse anti-β-actin antibody (A5316; 1:5,000; Sigma-Aldrich; 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-CB-CD antibody (3008; 1:200; Abcam, Cambridge, UK), mouse anti-TK antibody (sc-53331; 1:200; Santa Cruz Biotechnology, Inc.), rabbit anti-CD antibody (10348-924; 1:200; VWR (2.5 mM), 0.25 µl LA Taq polymerase and 1 µl 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. The reaction mixture for PCR contained the following: 0.25 µl P1 (10 µM), 0.25 µl P2 (10 µM), 19.75 µl dH2O, 2.5 µl 10X LA PCR buffer (Mg2+ Plus), 1 µl dNTPs (2.5 mM), 0.25 µl LA Taq polymerase and 1 µl 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 were digested by MluI and NheI respectively at 37˚C overnight, and the two linear fragments were connected at 4˚C overnight to construct pYr1.1-hTERTp-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 target expression. The hTERTp was synthesized by Yrbo, according to a previous study (21), and the sequence was: 5'-AGCCGTGCTCCAGTGATTTCGCGGACA GCAGCCCGAGCCCGCTCACCAGTGGCGGAN GAATGCGGGACCCGGGACCCGGTCTCGCTCCCTAC CTTCAGCTCGCCTCCCCGCGGAGATCCGCGGCCC GTGCCGACACCTCGGCGGGCCAGCCCGGTCTCG CCGGCGCTCAGGCGTCTCCCTCTCTCTCTCTCRTCCG GCGCCGCCCTCTCTCGCGCGGCGAGTTTTCAGCCAGC GCTCGCGCTCTGGACGACGACGGCCTGGATCGGCACC CCCGGCCACCCCAGCGGCTAGCC-3' (the underlined sections were MluI and NheI restriction sites, respectively), and was subcloned into pUC57 vector, which was named pUC57-hTERTp. pUC57-hTERTp and pYr1.1 were digested by MluI and NheI 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 GCTAGCCGCAACATGGTGACAGGGAATGGCA-3’ (NheI restriction site was introduced), and P2, 5’-CCGCTC GAGTACGTAGGCTCTCCCCCCATCT-3’ (XhoI restriction site was introduced). The reaction mixture for PCR contained the following: 0.25 µl P1 (10 µM), 0.25 µl P2 (10 µM), 19.75 µl dH2O, 2.5 µl 10X LA PCR buffer (Mg2+ Plus), 1 µl dNTPs (2.5 mM), 0.25 µl LA Taq polymerase and 1 µl 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 NheI and XhoI 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 MluI and NheI 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 NheI and XhoI respectively at 37˚C overnight, and the two linear fragments were connected (at 4˚C overnight) to construct
a novel triple-gene vector pYr1.1-hTERTp-yCDglyTK-shSurvivin2. Plasmids used in the current study are presented in Table II.

**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-shSurvivin2 | CDTK-shSurvivin2 | 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; shSurvivin, Survivin-targeted small hairpin RNA.

**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'-GGGAGATTAGAGGGCAAA GTGT-3' and reverse primer 5'-ACGGCGTCGTCAAGCGCAG GCGCCTTTC-3'. For Survivin, a PCR product of 107 bp was produced.

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**Table I. Sequences of oligonucleotides encoding Survivin-shRNA.**

| Survivin-shRNA | Sequences of oligonucleotides |
|---------------|-------------------------------|
| Survivin-sh1  | Forward: 5'-CACCGAGGCTGGCTTCCATCACCAGGCAACTGAGCAGTTGATGAAGCCAGCCTCTTTTTTG-3' | Reverse: 5'-AGCTCAAAAAAAAAGAGGCTGGCTTCCATCACCAGGCAACTGAGCAGTTGATGAAGCCAGCCTCTTTTTTG-3' |
| Survivin-sh2  | Forward: 5'-CACCGAGGCCAAGAACAATAATGCTTCAAGAGAGCAATTTGTTTCCTTTGCTCTTTTTTG-3' | Reverse: 5'-AGCTCAAAAAAAAAGAGGCTGGCTTCAAGAGAGCAATTTGTTTCCTTTGCTCTTTTTTG-3' |
| Survivin-sh3  | Forward: 5'-CACCGAAAGTGCAGCCTGCCATCTTCAGAGAGATGGCAGGCGACTTTCTTTTTTG-3' | Reverse: 5'-AGCTCAAAAAAAAAGAGGCTGGCTTCAAGAGAGCAATTTGTTTCCTTTGCTCTTTTTTG-3' |
by forward primer 5'-CATCTCTGCATCTGGACCTGG-3' and reverse primer 5'-TAATGTACGACGACGATTCC-3'. β-actin was used as an internal control, and the forward primer was 5'-AGCGGATCCATCCCCAAGTT-3' and the reverse primer was 5'-GGGCACGAAAGCTCATCTAT-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 Bandscan 5.0 (http://www.bbioo.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 × g for 15 min at 4°C. The supernatant was collected and protein concentrations were evaluated using a BCA protein assay. Identical amounts (40 µg 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 overnight at 4°C. The membranes were washed three times with PBS, and cultured in RPMI 1640 medium (supplemented with 10% FBS) with 200 µg/ml 5-FC and 16 µg/ml GCV was added when the cells reached 70% confluence. 48 h later, the cells were pelleted by centrifugation at 800 × 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 µg/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 5x10^5 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 µl 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 ± standard deviation. Statistical analysis was performed using SPSS version 13.0 (SPSS, Inc., Chicago, IL, 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 2x10^6 cells per flask. RPMI 1640 medium (supplemented with 10% FBS) with 200 µg/ml 5-FC and 16 µg/ml GCV was added when the cells reached 70% confluence. 48 h later, the cells were pelleted by centrifugation at 800 x 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 µg/ml) for 30 min at 37°C. Finally, the cell apoptosis rate was analyzed using flow cytometry (FACScanto, BD Biosciences; San Jose, CA, USA).

**Results**

Construction of the plasmid pYr1.1-hTERTp-γCDglyTK-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, γCDglyTK was cloned into pYr1.1-hTERTp to generate pYr1.1-hTERTp-γCDglyTK. Finally, Survivin-shRNA from pYr1.1-Survivin-sh2 was cloned into pYr1.1-hTERTp-γCDglyTK to develop the triple-gene plasmid pYr1.1-hTERTp-γCDglyTK-shSurvivin2. In this novel triple-expressing plasmid, the Survivin-shRNA sequence was driven by a U6 promoter, whereas fusion suicide gene γCDglyTK was regulated by hTERTp. The construction scheme of the triple-gene plasmid
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,
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.

CTDK-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±0.48%, in HCT/CDTK cells was 16.17±3.71% and in HCT/CDTK-shSur cells was 27.50±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.

CTDK-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).
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-F 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-F system is often adopted in treating gastrointestinal tumors (29). Double suicide gene combined with HSV-TK/GCV and CD/5-F system 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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