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Nanoscale bubble ultrasound contrast agents-mediated suicide gene therapy system, Nanoscale bubble-LV5-YCD-TK/GCV/5-FC, effectively inhibits bladder cancer cell growth

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Abstract. – OBJECTIVE: Bladder cancer is the 2nd most common reason for human genitourinary cancer-associated mortality. This study aimed to investigate the effects of Nanoscale bubble ultrasound contrast agents-mediated yeast-cytosine-deaminase-thymidine kinase/ganciclovir (YCD-TK/GCV) or YCD-TK/5-fluorocytosine (5-FC) suicide gene therapy system on BIU-87 cell growth.

MATERIALS AND METHODS: Targeted nanoscale bubble ultrasound contrast agents were prepared by utilizing thin-film hydration-sonication approach. Nanoscale bubble-LV5-YCD-TK/GCV(5-FC) was constructed and transfected to BIU-87 cells. Hematoxylin and eosin (HE) staining was used to evaluate inflammation. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay was used to examine cell viability. Cell-cycle distribution was analyzed with cell cycle assay. Flow cytometry assay was utilized to test apoptosis of BIU-87 cells. YCD-TK expression was examined using Western blot and quantitative Real Time-PCR (qRT-PCR), respectively.

RESULTS: YCD-TK highly expressed in Nanoscale bubble mediated suicide gene therapy system. Nanoscale bubble-mediated suicide gene therapy system significantly induced inflammatory response and apoptosis compared to that of Nanoscale bubble group ($p<0.05$). Nanoscale bubble mediated suicide gene therapy system significantly reduced cell viability compared to that of the Nanoscale bubble group ($p<0.05$). Nanoscale bubble mediated suicide gene therapy system significantly inhibited cell cycle arrest compared to that of the Nanoscale bubble group ($p<0.05$). Nanoscale bubble-LV5-YCD-TK/GCV/5-FC therapy system significantly reduced BIU-87 cell viability compared to that of the Nanoscale bubble-associated groups ($p<0.05$).

CONCLUSIONS: Nanoscale bubble-mediated suicide gene therapy system, bubble-LV5-YCD-TK/GCV/5-FC, acts as a novel therapeutic strategy for bladder cancer treatment.

Key Words
Bladder cancer, Nanoscale bubble, Suicide gene, Gene therapy.

Introduction

Bladder cancer is the 9th frequently occurred malignancy and 2nd most common reason for the human genitourinary cancer-associated mortality worldwide[1-3]. In the past decades, many therapeutic approaches have been applied for bladder cancer, such as surgery, radiotherapy, immunotherapy, chemotherapy; however, there are also plenty of side-effects and unfavorable outcomes[4,5]. Meanwhile, the high rate of recurrence and poor progression also affect the clinical management for superficial and muscle-invasive bladder cancer. Nowadays, the precision medicine and the multimodal treatments for cancer treatment have become the promising trend and been well-developed with favorable prognosis in recent years[6,7]. We speculated that enhancing the therapeutic efficacy or increasing the specificity may improve the clinical prognosis, by employing advanced drug-delivery strategies. Novel therapeutic modalities commonly used for drug-delivery of cancer treatment mainly include bio-adhesive microspheres, adenoviral vectors, magnetically targeted carries, anti-sense oligodeoxynucleotides[8,9]. Especially

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the adenoviral vectors, used for the gene therapy, have become a potential strategy for the clinical trials and laboratory experiments[10]. The recombinant adenovirus or the special replication-deficient adenovirus vector gene delivery system have been extensively applied for the tumorigenesis[11,12]. However, there are also a few shortcomings about the adenovirus-mediated gene therapy, such as the carrier toxicity, selectivity or specificity of adenovirus, which largely limits its limitation. The contrast-enhanced ultrasonography has remarkably improved the imaging of lesions, and the contrast agents have also been applied to the clinical practice for targeting the lesions[13,14]. In recent years, following the development of targeted ultrasound contrast agents and Nanoscale bubble (Nanobubble), Nanoscale bubble ultrasound contrast agents have appeared and led a revolutionary progress[15]. For the cancer therapy, its main procedure is to delivery the targeted Nanoscale bubble contrast agent carrying the specific gene, which could accumulate selectively in the tumor tissues or cells. The most specific cytotoxic gene therapy strategy is the carriers mediated transduction of the "suicide genes", which always encode the enzymes that could catalyze highly-toxic metabolites formation when interacted with the non-toxic pro-drugs. According to the previous studies[16,17], the herpes simplex virus thymidine kinase (HSV-TK) and yeast cytosine deaminase (YCD) are the most frequently used suicide genes. The best-known suicide gene therapeutic system is the HSV-TK combining Ganciclovir (GCV) or the YCD combining 5-fluorocytosine (5-FC)[16,18]. In this work, we explored the toxicity and efficacy of the nanoscale bubble ultrasound contrast agents-mediated YCD-TK/GCV or YCD-TK/5-FC with radiation therapy in the cultured bladder cancer cell line, BIU-87. Our results exhibited that Nanoscale bubble ultrasound contrast agents-mediated YCD-TK/GCV or YCD-TK/5-FC suicide gene therapy system could inhibit BIU-87 cell growth effectively.

Materials and Methods

Cell Culture

The human bladder cancer cell line, BIU-87, used in this study was purchased from the Cell Bank of the Chinese Academy of Science, Shanghai, China. The BIU-87 cells were cultured in the Roswell Park Memorial Institute-1640 (RPMI-1640, Gibco Ltd., Grand Island, NY, USA) supplementing with 10% fetal bovine serum (FBS, Gibco Ltd., Grand Island, NY, USA), 100 μg/ml streptomycin and 100 U/ml penicillin (Beyotime Biotech. Shanghai, China) in 5% CO_2 at 37°C and humidified environment (Figure 1A). The present study was approved by the Ethics Committee of Chongqing Medical University (Chongqing, China).

Nanoscale Bubble Ultrasound Contrast Agent Preparation

The targeted Nanoscale bubble ultrasound contrast agents were prepared by utilizing the thin-film hydration-sonication approach according to previous study described[19]. In brief, 1, 2-distearoyl-sn-glycero-3-polyethylene glycol-maleimide 2000 (DSPE-PEG2000, Shanghai Advanced Vehicle Technology, Ltd. Co., Shanghai, China), hydrogenated soy phosphatide (HSPC, Shanghai Advanced Vehicle Technology, Ltd. Co.), diphenylphosphoryl zaide (DPPA,

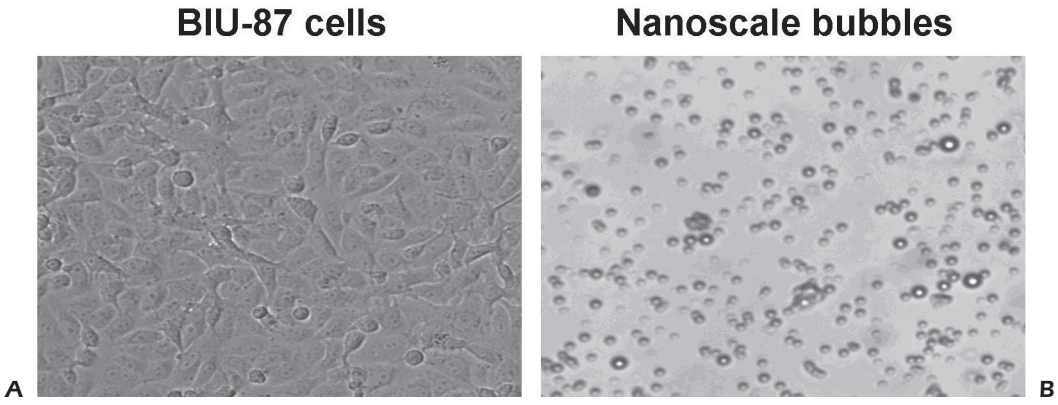

Figure 1. BIU-87 cell culture and Nanoscale bubbles preparation.

Shanghai Advanced Vehicle Technology, Ltd. Co.) were dissolved in the chloroform solution (1 ml, Sino Pharm. Group Co. Ltd., Shanghai, China). The obtained solution was evaporated to form the thin-film, which was hydrated using the hydrating liquid (1 ml, Bio-Rad Laboratories, Hercules, CA, USA), dried in a vacuum for 12 h and maintained in the shaking incubator at 55°C for 60 min to get the liposomes. Then, the liposomal was transferred to the Eppendorf tube (EP tube, 1.5 ml, Eppendorf, Hamburg, Germany), the air in which was substituted with the C_3F_8 gas (Sino Pharm. Group Co. Ltd., Shanghai, China). At last, the liposome was sonicated for 8 s at 95 W. The biotinylated lipid nanoscale bubbles were harvested post the large nanoscale bubbles were separated forming a thin-layer by centrifuging at low-speed. According to the previous study[20], the biotinylated lipid nanoscale bubbles were treated with streptavidin (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 4°C, and targeted nanoscale bubble ultrasound contrast agents were well acquired (Figure 1B).

Plasmid Construction, Lentivirus Packaging

pG-LV5 lentiviral vector (LV5) (GenePhama Co. Ltd, Shanghai, China) was used to construct the recombined LV5 plasmid carrying YCD and TK gene, which was named as LV5-YCD-TK. The oligonucleotides for the YCD and TK gene were constructed and synthesized by GenePhama Co. Ltd. (Shanghai, China). Finally, the synthesized double-chain YCD-TK gene sequences were sub-cloned into the pG-LV5 plasmid (Figure 2A) to form LV5-YCD-TK plasmid. The agarose gel electrophoresis and enzyme digestion results showed that the sequences of YCD and TK gene are corrected (Figure 2B). Then, the LV5-YCD-TK plasmid and packing plasmids, including PG-p1-VSVG, PG-P2-REV, PG-P3-RRE, were transfected using lentiviral packaging kit (Cat. No. 41102ES10, Yeast Biosci. Tech., Shanghai, China), according to the instruction of the manufacturer. The detailed processes for viral packaging were performed due to previous study described[21]. The titer of LV5-YCD-TK virus was

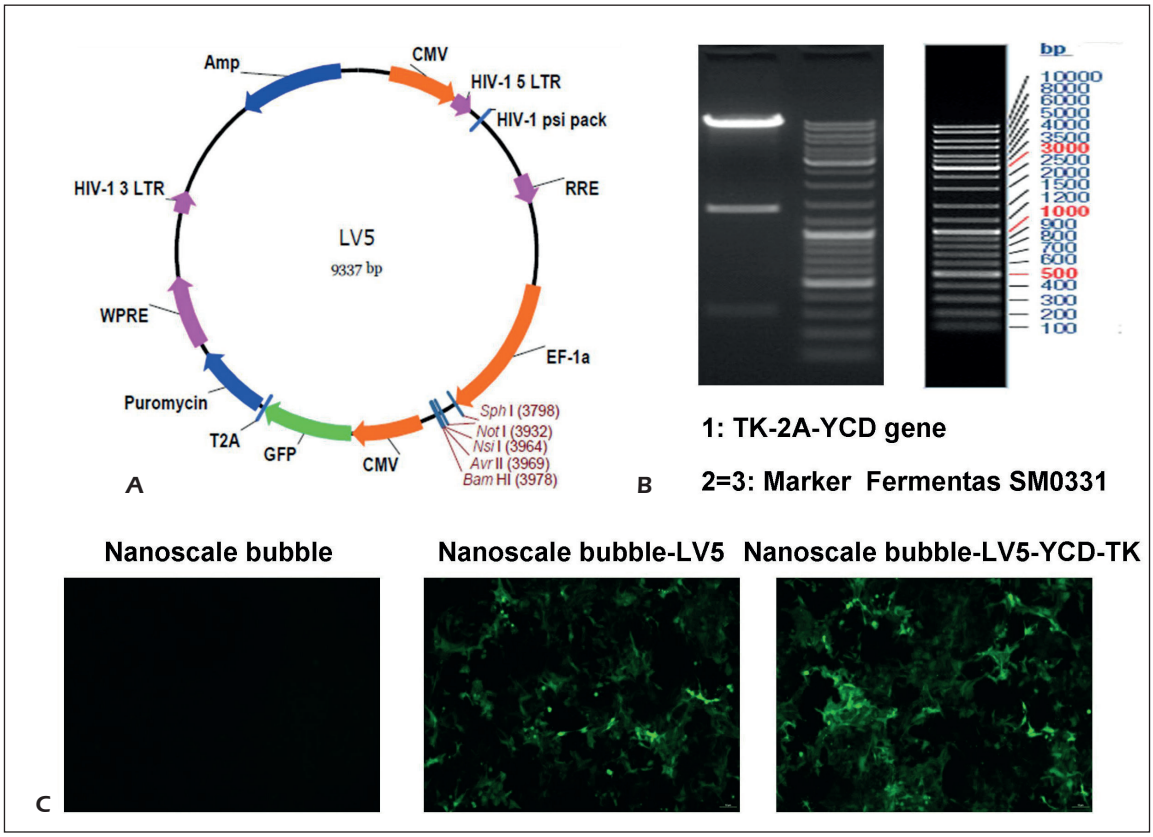

Figure 2. LV5-YCD-TK plasmid construction and efficacy evaluation. **A**, Blank p-LV5 vector image. **B**, Amplification and enzyme digestion of targeted YCD-TK gene. **C**, Evaluation for the efficacy of LV5-YCD-TK plasmid transfection.

evaluated by examining GFP expression due to the manufacturer's instructions.

Transfection of Nanoscale Bubble Ultrasound Contrast Agents

The BIU-87 cells were cultured as the above described and passaged for 3-4 generations. Nanoscale bubble ultrasound contrast agents were added to the BIU-87 cells and adjusted to the concentration of 0.1 μg/μl. Then, the LV5 vector carrying the YCD gene and TK gene (LV5-YCD-TK) was transfected into the BIU-87 cells using Nanoscale bubble ultrasound contrast agents and sonicated for 60 s (with the cycle of 4 s for ultrasonic processing and 20 s of the interval). Finally, the G418 (at a concentration of 400 μg/μl) was used to screen the positive transfection BIU-87 cells. The Nanoscale bubble-LV5-YCD-TK was successfully transfected into the BIU-87 cells and exhibited higher transfection efficacy (Figure 2C), which were used for the following experiments.

Hematoxylin and Eosin (HE) Staining

The BIU-87 cells were incubated with 4% formaldehyde (Beyotime Biotech. Shanghai, China) in Phosphate-Buffered Solution (PBS, Beyotime Biotech. Shanghai, China). The histology was visualized using HE staining by using both of hematoxylin (Nanjing Jiancheng Bio. Engineering Institute, Nanjing, China) and eosin (Beyotime Biotech. Shanghai, China) according to the previous study described[22]. HE-stained BIU-87 cells were captured with a digital microscope (Mode: DSX110, Olympus, Tokyo, Japan). The digital images for HE staining were collected from the representative areas with the magnification of 200 ×.

3-(4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyl-2-H-Tetrazolium Bromide (MTT) Assay

The viability of BIU-87 cells was evaluated by employing a MTT assay according to the previous study reported[23]. Briefly, BIU-87 cells were seeded into 96-well plates (Corning Inc., Corning, NY, USA) and cultured in the condition 37°C and of 5% CO_2. Then, the BIU-87 cells were incubated with MTT solution (Sigma-Aldrich, St. Louis, MO, USA, at a concentration of 5 mg/ml) at 37°C for 4 h. Finally, 150 μl dimethyl sulfoxide (DMSO, Amresco Inc., Solon, OH, USA) was added into the 96-well plates and incubated for 10 min to dissolve the crystal. Optical density (OD) values were examined using enzyme-linked immunosorbent assay (ELISA) reader (Thermo Fisher Scientific, Waltham, MA, USA) at a wavelength of 490 nm. The cell viability was calculated according to the obtained OD values.

Cell Cycle Analysis

Cell cycle distribution of BIU-87 cells was evaluated by using Cell Cycle and Apoptosis Analysis kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instruction. In brief, BIU-87 cells were harvested, washed three times with PBS and fixed with 70% ethanol (Beyotime Biotech. Shanghai, China) at 4°C for 1 h. Then, BIU-87 cells were stained with the Annexin V (BD Biosciences, Franklin Lakes, NJ, USA) and propidium iodide (PI; BD Biosciences, Franklin Lakes, NJ, USA) solution (containing RNase), respectively, at 4°C for 30 min. 20000 cells were analyzed using FACS Vantage SE flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). Cell cycle distribution of BIU-87 cells was analyzed with the ModFIT cell cycle analysis software (Version 2.01.2; BD Biosciences, Franklin Lakes, NJ, USA).

Flow Cytometry Assay

Apoptosis of BIU-87 cells was evaluated by using the Annexin V-PE/7-AAD apoptosis kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the instruction of the manufacturer. Briefly, BIU-87 cells were harvested and suspended in the Annexin V binding buffer (BD Biosciences, Franklin Lakes, NJ, USA) and incubated with Annexin V-PE and PI (BD Biosciences, Franklin Lakes, NJ, USA) in the dark for 15 min at room temperature. The BIU-87 cells were analyzed by employing the FACS Vantage SE flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). Finally, the fluorescence of staining cells was examined with a 546/647 band filter to monitor PI and with a 530/578 band filter to monitor Annexin V binding in the BIU-87 cells.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNAs of BIU-87 cells were extracted with the commercial reagents (Sino Pharm. Chem. Reagent Co. Ltd., Shanghai, China) with the TRIzol method according to manufacturer's instructions. The complementary DNA (cDNA) was synthesized by utilizing the Reverse Transcription Kit (Western Biotech., Chongqing,

Table I. The primers for the RT-PCR assay.

	Gene	Sequences	Length (bp)
YCD-TK	Forards	5'-GTATCCCAACGGTGAAGCG-3'	127
	Reverse	5'-CAGGGCGAAGGTTTTATGC-3'	
β-actin	Forards	5'-TGACGTGGACATCCGCAAAG-3'	205
	Reverse	5'-CTGGAAGGTGGACAGCGAGG-3'	

China) according to manufacturers' instruction. SybrGreen PCR master Mix (Western Biotech., Chongqing, China) was used to amplify the YCD-TK and β-actin genes. The primers used for qRT-PCR were listed in Table I. The thermal cycling (CT) parameters for quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) were listed as the following: denaturation step at 94°C for 4 min, followed by 35 cycles of amplification of 94°C for 20 s, 60°C for 30 s, and 72°C for 30 s. The relative gene expression target YCD-TK was calculated with GDS8000 gel scanning system for Real Time-PCR (UVP corporation, Sacramento, CA, USA) by using the formula of $2^{-\Delta\Delta ct}$.

Western Blot Assay

The BIU-87 cells were lysed by using the radioimmunoprecipitation assay (RIPA) and the products were centrifuged at 10000 ×g for 10 min. The supernatant of BIU-87 cells was separated using the 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Amresco Inc., Solon, OH, USA) and then transferred onto polyvinylidene difluoride membrane (PVDF, Bio-Rad Laboratories, Hercules, CA, USA) with Trans-Blot SD cell instrument (Bio-Rad Laboratories, Hercules, CA, USA). Post the electronic-transfer, the PVDF membranes were incubated with the 5% non-fat dry milk (BD Biosciences, Franklin Lakes, NJ, USA) at room temperature for 2 h. PVDF membranes were then incubated with the rabbit anti-human TK polyclonal antibody (1: 2000; Cat. No. ab129880, Abcam Biotech., Cambridge, MA, USA) and rabbit anti-human β-actin antibody (1: 3000; Cat. No. ab8226, Abcam, Cambridge, MA, USA) at 4°C overnight. The polyvinylidene difluoride (PVDF) membranes were then washed with Phosphate-Buffered Saline and Tween-20 (PBST) and incubated by using horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (1:2000, Sigma-Aldrich, St. Louis, MO, USA) and HRP-labeled goat anti-mouse IgG (1:2000, Sigma-Aldrich. St. Louis, MO, USA) at 37°C for 60 min. The enhanced chemiluminescence (ECL, Thermo Fisher Scientific, Waltham, MA, USA) was used to treat the polyvinylidene difluoride (PVDF) membrane for 2 min in the dark. The relative grey density was analyzed using the Labworks™ Analysis Software (Labworks, Upland, CA, USA).

Statistical Analysis

Data were described as mean ± standard deviation (SD) and analyzed with SPSS software 19.0 (SPSS Inc., Chicago, IL, USA). The Student's *t*-test was used for statistical analysis between the two groups. Tukey's post hoc test was used to validate the analysis of variance (ANOVA) for comparing the data among groups. All of the data were obtained from at least six independent experiments or test. A statistical significance was defined when $p<0.05$.

Results

YCD-TK Highly Expressed in Nanoscale Bubble Mediated Suicide Gene Therapy System

To confirm the expression of YCD-TK in BIU-87 cells undergoing Nanoscale bubble ultrasound contrast agents, the qRT-PCR and Western blot assay were used in this study. The qRT-PCR results showed that the YCD-TK mRNA levels in Nanoscale bubble-LV5-YCD-TK group were higher compared to that of the Nanoscale bubble and Nanoscale bubble-LV5 group (Figure 3A, $p<0.05$). Meanwhile, the Western blot assay results indicated that the Nanoscale bubble-LV5-YCD-TK transfection significantly enhanced the levels of YCD-TK compared to Nanoscale bubble or Nanoscale bubble-V5 transfection group (Figure 3B, $p<0.05$).

Nanoscale Bubble Mediated Suicide Gene Therapy System Induced Inflammatory Response

The inflammation could reflect the cell homeostasis, therefore, the inflammatory response in BIU-87 cells undergoing Nanoscale bubble-LV5-YCD-TK transfection was evaluated using

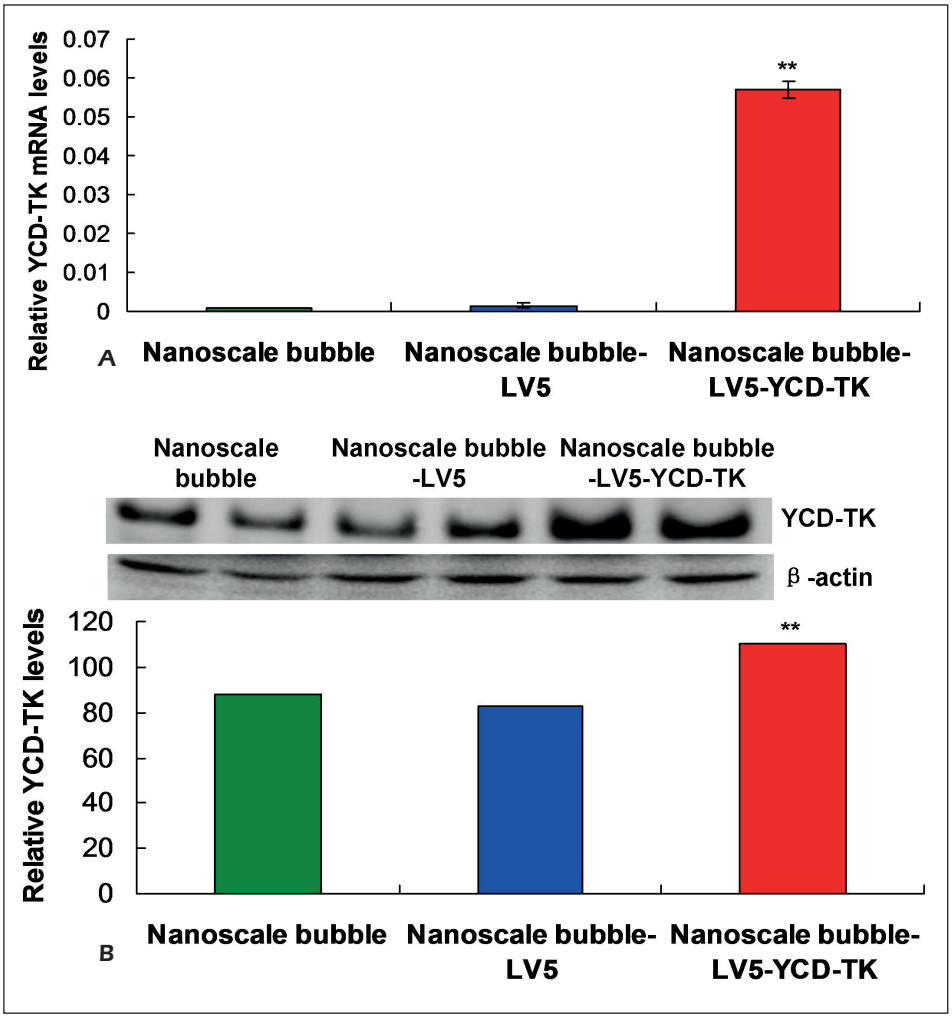

Figure 3. Evaluation for the YCD-TK mRNA and protein expression by using qRT-PCR and Western blot assay, respectively. **A**, qRT-PCR assay for YCD-TK mRNA expression. **B**, Western blot assay for YCD-TL protein expression. $^{**}p<0.01$ *vs.* Nanoscale bubble group.

HE staining (Figure 4A). The results showed that the amounts of inflammatory cells in the Nanoscale bubble-LV5-YCD-TK group were significant compared to that of Nanoscale bubble or Nanoscale bubble-LV5 group (Figure 4B, $p<0.05$).

Nanoscale Bubble Mediated Suicide Gene Therapy System Reduced Cell Viability and Induced Apoptosis

To observe the effects of Nanoscale bubble-mediated suicide gene therapy system therapy on the cell growth, the MTT and flow cytometry assay was used to evaluate cell viability and apoptosis, respectively. The MTT results indicated that Nanoscale bubble-LV5-YCD-TK transfection significantly reduced the BIU-87 cell viability at 24 h, 36 h, 48 h and 72 h, compared to the Nanoscale bubble LV-5 and Nanoscale bubble group (Figure 5A, $p<0.05$). Also, the Nanoscale bubble-LV5-YCD-TK transfection significantly reduced the BIU-87 cell apoptosis compared to that of Nanoscale bubble and Nanoscale bubble LV-5 group (Figure 5B, $p<0.05$).

Nanoscale Bubble Mediated Suicide Gene Therapy System Inhibited Cell Cycle Arrest

Cell cycles of BIU-87 cells undergoing Nanoscale bubble mediated suicide gene therapy system treatment also monitored by flow cytometry (Figure 6A). As demonstrated in Figure 6B, Nanoscale bubble-LV5-YCD-TK transfection resulted in more cells in the G1 phase compared to the Nanoscale bubble group ($p<0.05$). Meanwhile, cells in the G2 phase of Nanoscale bubble-LV5-YCD-TK transfection group were fewer compared to the Nanoscale bubble group (Figure 6B, $p<0.05$). Moreover, the BIU-87 cells in the S-phase were significantly lower in Nanoscale bubble-LV5-YCD-

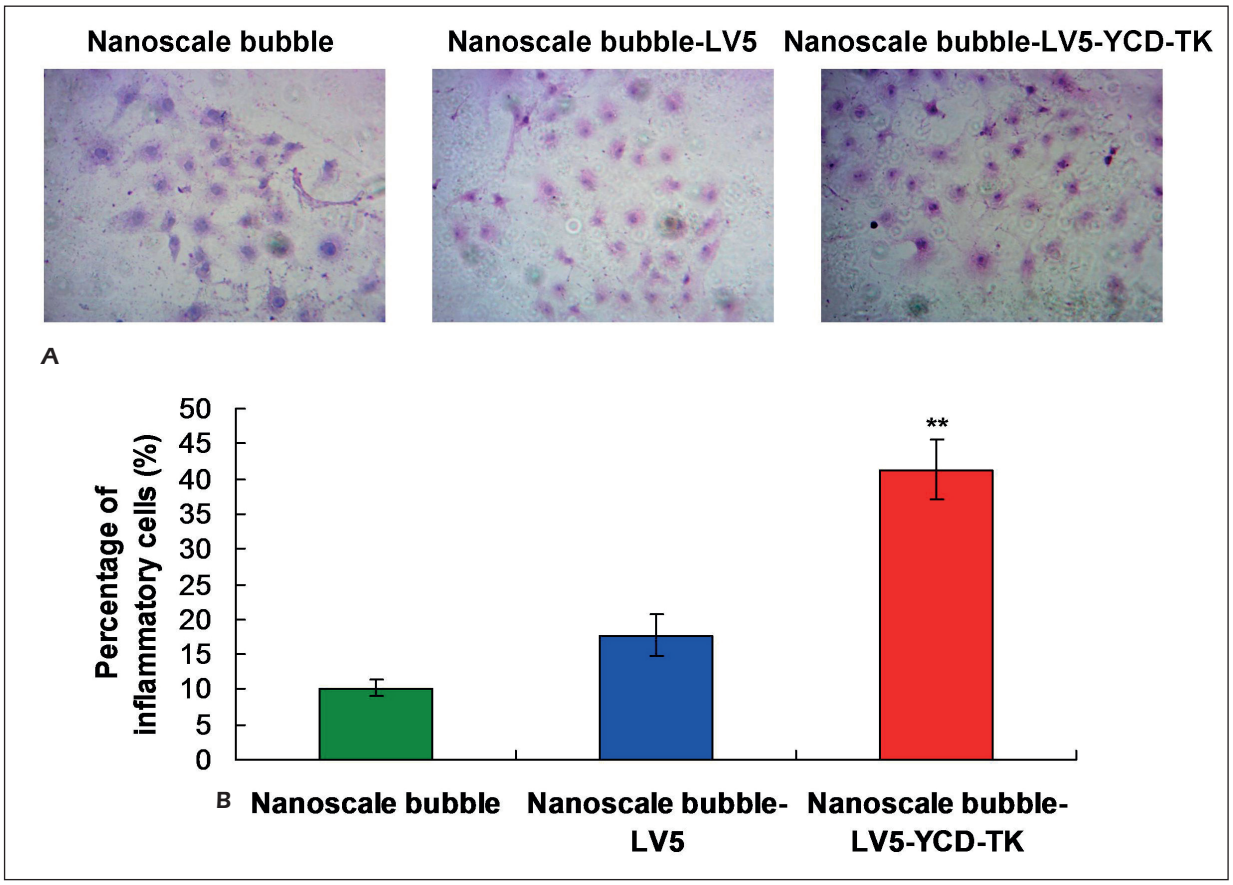

Figure 4. HE staining for examining the inflammatory response in BIU-87 cells. A, Image for the HE staining of BIU-87 cells. B, Statistical analysis for the HE staining. **$p<0.01$ *vs.* Nanoscale bubble group.

TK transfection cells compared to that in the Nanoscale bubble group (Figure 6B, $p<0.05$).

Nanoscale Bubble-LV5-YCD-TK/GCV/5-FC Therapy Reduced BIU-87 Cell Viability

To evaluate the anti-tumor activity of the Nanoscale bubble as a novel gene transfer vehicle, we examined the cytotoxic-effects of Nanoscale bubble-LV5-YCD-TK combining with GCV or/and 5-FC treatment on BIU-87 cells. The results showed that the cell viabilities in GCV or 5-FC group were lower compared to that in the group without drug treatment (Figure 7, $p<0.05$), in Nanoscale bubble, Nanoscale bubble-LV5 and Nanoscale bubble-LV5-YCD-TK transfected BIU-87 cells. What's most important is that the cell viabilities of Nanoscale bubble-LV5-YCD-TK/GCV or Nanoscale bubble-LV5-YCD-TK/5-FC or Nanoscale bubble-LV5-YCD-TK/GCV/5-FC group were lower compared to that of Nanoscale bubble/GCV or Nanoscale bubble-LV5/GCV, Nanoscale bubble/5-FC or Nanoscale bubble-LV5/5-FC and Nanoscale bubble/GCV/5-FC or Nanoscale bubble-LV5/GCV/5-FC group, respectively (Figure 7, $p<0.05$). Moreover, the Nanoscale bubble-LV5-YCD-TK/GCV/5-FC group significantly reduced the BIU-87 cell viability compared to that of the single Nanoscale bubble-LV5-YCD-TK/GCV or Nanoscale bubble-LV5-YCD-TK/5-FC group (Figure 7, $p<0.05$).

Discussion

At present, the gene therapy-dependent precision medicine has been considered to be a potential and promising strategy for tumor treatment[24]. As the proteins or nucleic acids can't penetrate the cell membrane, several viruses, including herpes simplex viruses, lentiviruses,

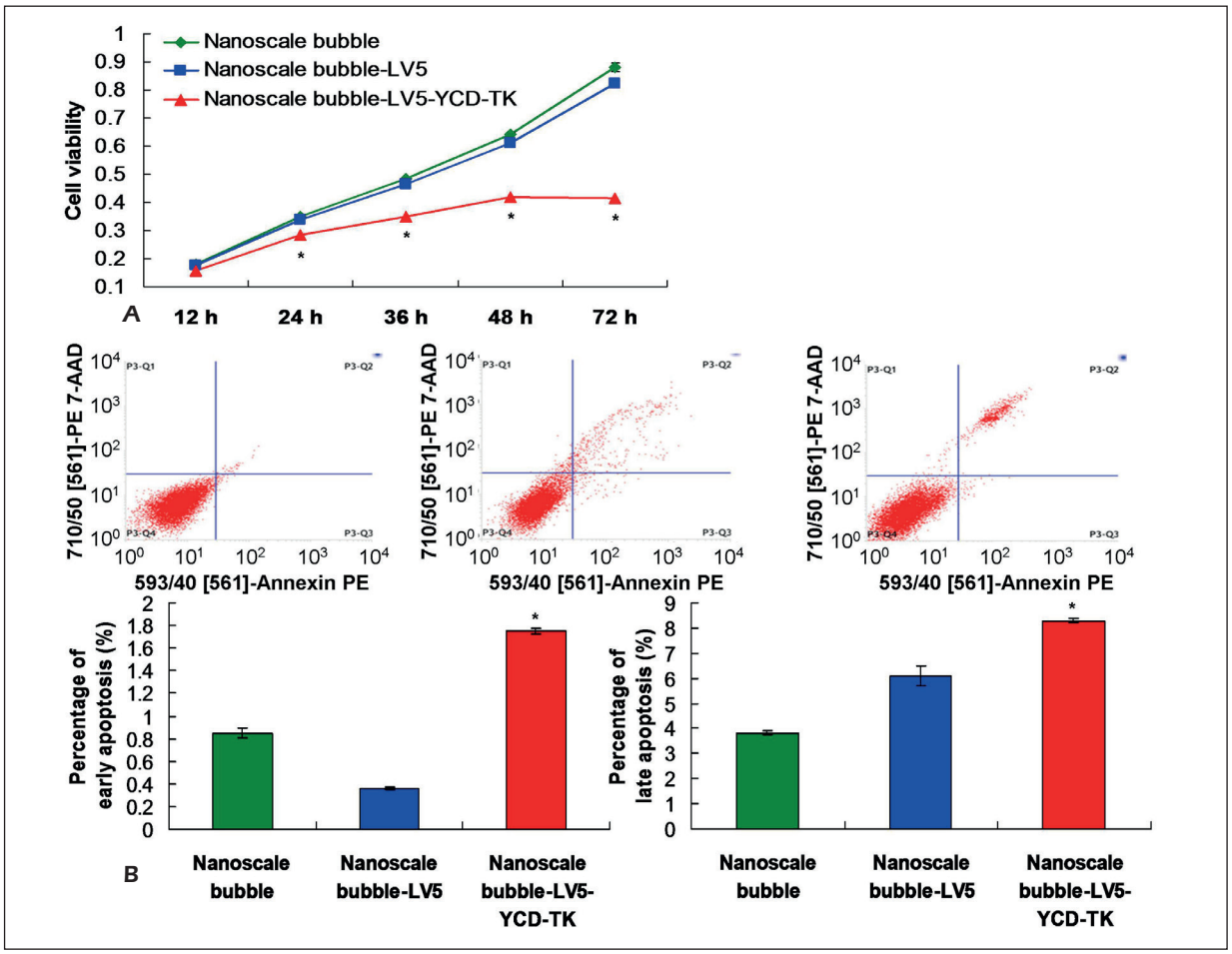

Figure 5. Analysis for the cell viability and apoptosis of BIU-87 cells. **A**, Cell viability examination by using MTT assay. **B**, Apoptosis analysis by using flow cytometry assay. *$p<0.05$ *vs.* Nanoscale bubble group.

retroviruses and adenoviruses, have been commonly applied to be the gene-delivery carriers[25]. Although the above virus vectors have been extensively used for *in vivo* or *in vitro* experiments, which also exhibit a few limitations, such as cell cytotoxicity, lower cell selectivity and poor specificity, in recent years, the nanoscale ultrasound contrast-enhanced agents with phospholipids, polymers, liquid, gas have been fabricated and exhibited good contrast enhancement ability[26,27]. However, the previous study mainly focused on the effects of Nanoscale ultrasound contrast-enhanced agents on enhancement ability in normal organs or lesions, but not no effects on tumor targeting. Therefore, in this work, we attempted to utilize the fabricate nanosized Nanoscale ultrasound contrast-enhanced agents together with suicide gene, YCD-TK, to target the tumor lesions. A cytotoxic gene therapy strategy is the suicide gene, herpes simplex TK gene following with the systemic GCV administration, which has been used in many tumor models to inhibit the tumor growth[28,29]. The tumor tissues expressed TK can efficiently convert the pro-drug GCV to the GCV-3-phosphate, which could kill the cancer cells[3]. Therefore, we constructed the Nanoscale bubble-LV5-YCD-TK/GCV therapy system in this study. Meanwhile, the suicide gene, YCD, can convert the pro-drug 5-FC to the 5-fluorouracil, combining 5-FC illustrates radio-sensitizing and chemo-therapeutic activity in a few cancers[30]. We also constructed a Nanoscale bubble-LV5-YCD-TK/5-FC therapy system. Inflammation is mainly characterized by infiltration, migration and activation of the leukocytes[31], which was evaluated in the bladder cancer cell line, BIU-87 cell, using HE staining. Our results showed

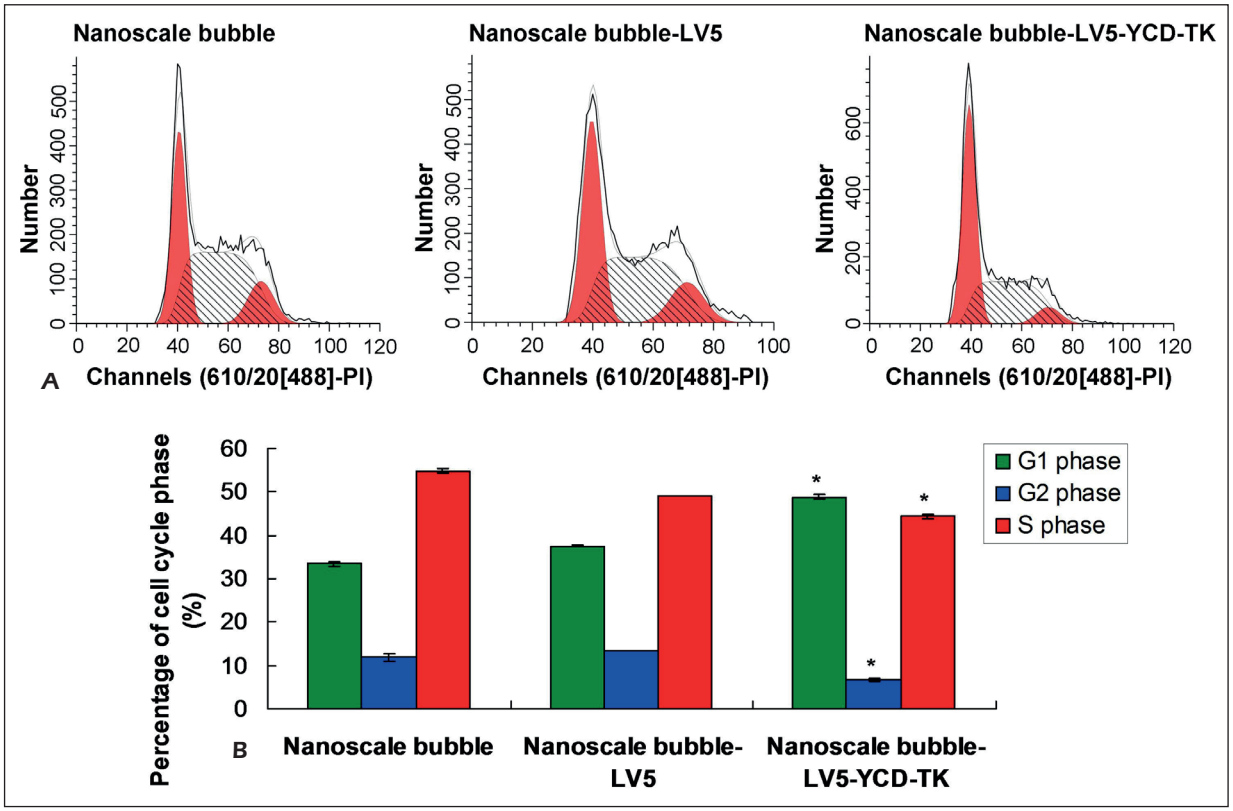

Figure 6. Cell cycle phase distribution evaluation by using flow cytometry assay. **A**, Images for the flow cytometry assay. **B**, Statistical analysis of the flow cytometry assay data. *p<0.05 *vs.* Nanoscale bubble group.

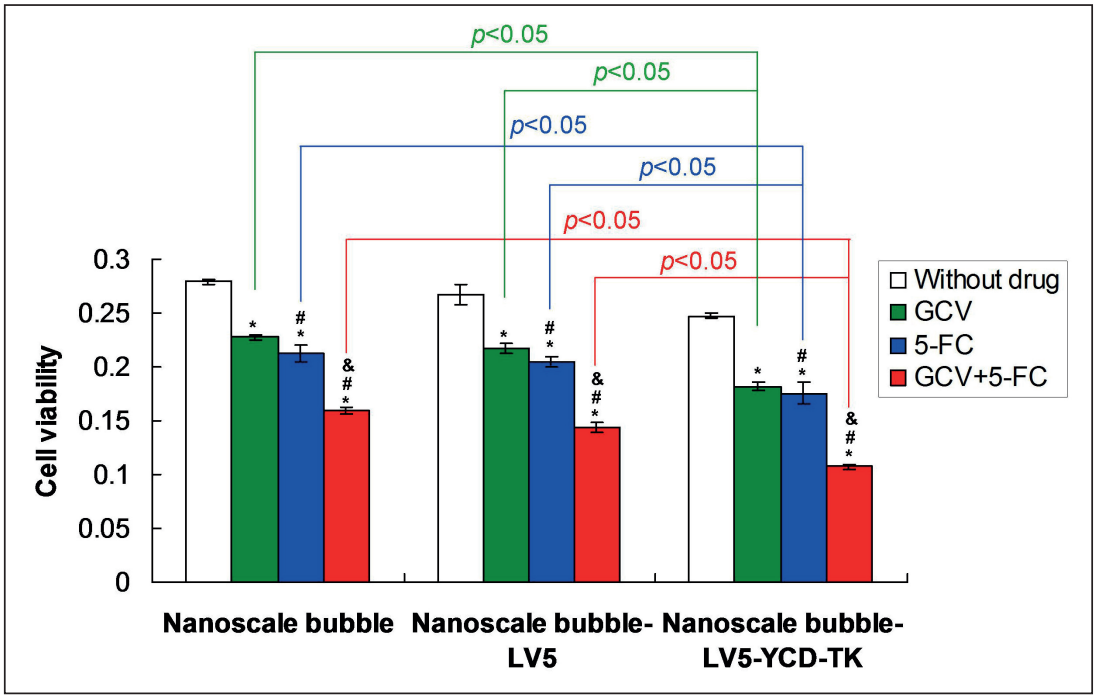

Figure 7. Cell viability evaluation of BIU-87 cells undergoing Nanoscale bubble-LV5-YCD-TK/GCV/5-FC suicide gene therapy system treatment. *p<0.05 vs. without drug group, $^{\#}p$<0.05 *vs.* GCV group, $^{\&}p$<0.05 *vs.* 5-FC group.

that the amounts of inflammatory cells in Nanoscale bubble-LV5-YCD-TK group were more significant compared to that of Nanoscale bubble or Nanoscale bubble-LV5 group, which suggest that Nanoscale bubble-LV5-YCD-TK transfection induced infiltration of leukocytes and BIU-87 cell damages. We also examined the BIU-87 cell viability using the MTT assay, the results of which indicated that Nanoscale bubble-LV5-YCD-TK infection significantly reduced the cell viabilities compared to that of Nanoscale bubble and Nanoscale bubble-LV5 group. This result hints that the Nanoscale bubble-LV5-YCD-TK treatment may induce the apoptosis of the BIU-87 cells. As known, the apoptosis is a critical cause for the tumor cell death[32]; therefore, the apoptosis of BIU-87 cells was evaluated. The flow cytometry assay results showed that Nanoscale bubble-LV5-YCD-TK infection significantly induced the early apoptosis and late apoptosis compared to that of the Nanoscale bubble or Nanoscale bubble-LV5 group. These results suggest that the suicide gene, YCD-TK, effectively targeted to the tumor lesions and played critical roles in BIU-87 cell death and apoptosis. These findings were consistent with the previously published studies that administrating the YCD or TK gene *in vivo* or *in vitro* experiments by combining with the other suicide gene delivery system, such as replication-competent adenovirus[33], fiber-modified adenovirus[12], *Bifidobacterium infantis*[3], immunogenic human-papillomavirus pseudovirus[34]. The cell cycle assay also indicated that Nanoscale bubble mediated suicide gene therapy system significantly inhibited cell cycle arrest by decreasing S phase and G2 phase cells, which is consistent with the apoptosis of BIU-87 cells. Wang et al[12] reported that the herpes simplex viruses-TK illustrated about 1000 folds of affinity to the pro-drug GCV compared to mammalian TK. In our work, we used the Nanoscale bubble mediated suicide gene therapy system combining with GCV or 5-FC as the therapeutic strategy and observed effects of which on BIU-87 cell viabilities. Our results illustrated that Nanoscale bubble-LV5-YCD-TK/GCV, Nanoscale bubble-LV5-YCD-TK/5-FC and Nanoscale bubble-LV5-YCD-TK/GCV/5-FC therapy all significantly reduced the BIU-87 cell viabilities compared to that of the Nanoscale bubble without combining with YCD-TK gene. Moreover, Nanoscale bubble-LV5-YCD-TK/GCV/5-FC group significantly reduced the BIU-87 cell viability compared to that of single Nanoscale bubble-LV5-YCD-TK/GCV or Nanoscale bubble-LV5-YCD-TK/5-FC group. These results suggest that the Nanoscale bubble-LV5-YCD-TK suicide gene therapy system effectively activated the pro-drugs, GCV and 5-FC, and remarkably inhibited the BIU-87 cell growth.

Conclusions

We successfully established a novel Nanoscale bubble mediated suicide gene therapy system to inhibit the bladder cancer cells growth. The Nanoscale bubble-LV5-YCD-TK can efficiently decrease the BIU-87 cell viability, induce BIU-87 cell apoptosis and suppress cell cycle arrest. When the Nanoscale bubble-LV5-YCD-TK/GCV/5-FC suicide gene therapy system was transfected into BIU-87 cells, the therapy system could significantly inhibit BIU-87 cell growth. Therefore, the present results suggest that Nanoscale bubble mediated suicide gene therapy system would act as a novel therapeutic strategy for the bladder cancer.

Conflict of Interests

The authors declare that they have no conflict of interest.

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