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Gemcitabine Conjugated Chitosan and Double Antibodies (Abc-GC-Gemcitabine Nanoparticles) Enhanced Cytoplasmic Uptake of Gemcitabine and Inhibit Proliferation and Metastasis In Human SW1990 Pancreatic Cancer Cells

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Pancreatic cancer is considered a chemoresistant neoplasm with extremely dismal prognosis and gemcitabine treatment is associated with many side effects and poor overall survival. The study aimed at developing a new nanobioconjugate, which specifically delivered gemcitabine and anti-EGFR antibody into pancreatic cancer cells.


Material/Methods: The novel nanodrug is based on chitosan platform, which is non-toxic, biocompatibility and biodegradable. We measured the effects of proliferation and metastasis on SW1990 by CCK-8 assay, colony formation assay, wound healing assay and Transwell assay. The expression of related proteins were evaluated by Western blot.

Results: We synthesized Abc-GC-gemcitabine nanoparticles successfully with the encapsulation rate of nanobioconjugates was 91.63% and the drug loadings was 9.97%. Both GC-gemcitabine microspheres solution (GC group) and Abc-GC-gemcitabine microspheres solution (Abc group) inhibited cells proliferation, colony formation, migration and invasion in SW1990 cells dramatically. Moreover, Abc-GC-gemcitabine microspheres expressed more significant inhibited action than GC-gemcitabine microspheres efficiently

Conclusions: Our data suggested that Abc-GC-gemcitabine nanoparticles could have promising potential in treating metastasized and chemoresistant pancreatic cancer by enhancing the drug efficacy and minimizing off target effects.

MeSH Keywords: **Cell Proliferation • Geminiviridae • Nanoparticles • Neoplasm Metastasis • Pancreatic Neoplasms**

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Background

Pancreatic cancer is one of the deadliest cancers worldwide [1]. The overall 5-year survival rate is less than 5% and the prognosis of pancreatic cancer remains extremely poor [2]. Surgical resection is the only curative therapeutic treatment for this disease. However, given the concealed location, pancreatic cancer is often not discovered until serious clinical symptoms and signs are present, which means only the minority of patients can be resected [3,4]. In most cases, the vast majority of pancreatic cancer patients prefer to chemotherapy.

Gemcitabine (GEM, 2',2'-difluorodeoxycytidine) is a nucleotide analogue widely used in cancer treatment [5]. At present, systemic gemcitabine-based chemotherapy has been used as the standard therapy for patients with advanced pancreatic cancer [6]. However, this treatment is associated with many side effects and poor overall survival, and the therapeutic efficacy of pancreatic cancer are still far from satisfaction [7]. In order to improve the overall survival of patients with pancreatic cancer, many studies combine the use of gemcitabine with different agents [8–10].

EGFR (epidermal growth factor receptor-1) is a member of the EGFR/ErbB/HER family of type I transmembrane tyrosine kinase receptors [11,12]. High expression of EGFR induces erroneous development and unrestricted proliferation in a number of human malignancies, including pancreatic cancer [11]. Tumors overexpressing EGFR tend to have increased cell proliferation, more rapid cell cycle progression, inhibition of apoptosis, and higher rates of metastasis [13]. Therefore, EGFR is a potential therapeutic target for the successful treatment of pancreatic cancer.

Recently, considerable attention has been directed toward nanotechnologies; nanotechnologies are the design, characterization, production, and application of structures, devices, and systems by controlling shape and size at the nanometer scale [14,15]. The application of nanotechnologies to pharmaceutical research and development has led to the successful development of nanodrugs [14]. When designed with drugs encapsulated in a carrier, the nanodrug delivery system demonstrated significantly higher antitumor activity in primary and metastatic cancers compared to drug alone and a PEGylated anticancer agent [16].

In this study, we aimed to develop a new nanobioconjugate which specifically delivered gemcitabine and anti-EGFR antibody into pancreatic cancer cells and efficiently inhibited tumor growth and metastatic. The novel nanodrug is based on chitosan platform, which is non-toxic, biocompatibility, and biodegradable [17].

Material and Methods

Ethylene glycol chitosan nanoparticle preparation

Glycol chitosan (GC) (Sigma Aldrich, St. Louis, MO, USA) was dissolved in distilled water and mixed at a constant temperature in a magnetic stirrer for 3 hours at a constant speed. Different concentrations of sodium tripolyphosphate (TPP) aqueous solution were added into the mixed solution and treated with probe type ultrasonic processor. The nanoparticle suspension solution was obtained when obvious opalescence was observed [18].

Synthesis of glycol chitosan nanobioconjugates

The following steps were executed in dark conditions. First, 13.46 mg aconitic acid anhydride (Qifa Biotech, Shanghai, China) were dissolved in 1 mL dioxane (Qifa Biotech, Shanghai, China), and 10 mg gemcitabine (Qifa Biotech, Shanghai, China) were dissolved in 400 μ L pyridine (Qifa Biotech, Shanghai, China). Then aconitum anhydride solution was slowly added dropwise into the gemcitabine solution and stirred overnight at 4°C. This was followed by washing twice in 5 mL chloroform and 5 mL 5% sodium bicarbonate. The remaining solution were extracted with ethyl acetate solution (Qifa Biotech, Shanghai, China), dried in a vacuum to obtain cis-aconitum acyl gemcitabine. Then 100 mg GC was dissolved in 10 mL distilled water and diluted with 10 mL methanol. Then cis-aconitum acyl gemcitabine was added into GC solution slowly and then stirred slowly with 1-baton rouge-3-(3-dimethylamino-propyl) carbodiimide (EDC) (Qifa Biotech, Shanghai, China) and N-hydroxysuccinimide (NHS) (Qifa Biotech, Shanghai, China) overnight at room temperature. Acquired products of GC-gemcitabine were preserved by freeze-drying after being dialyzed by 12,000 intercept molecular weight dialysis membrane.

The preparation of antibody complex (Abc)-GC-gemcitabine nanoparticles were as follow: combined the prepared GC-gemcitabine nanoparticles with the chitosan antibody and anti-EGFR antibody (Santa Cruz, Shanghai) under the condition of 37°C water bath.

Determination of the drug-loading

We calculated the encapsulation rate and drug loadings according to the following formula [19]: drug loadings = [coagulant dosage (MBq) – residual liquid dosage (MBq)] / nanoparticles (g) \times 100%; encapsulation rate = [coagulant dosage (MBq) – residual liquid dosage (MBq)] / coagulant dosage (MBq) \times 100%.

Analysis of drug release curve *in vitro*

500 mL GC-gemcitabine microspheres solution at a concentration of 2 mg/mL were put in trace dialysis tubes, which were suspended within 20 mL of distilled water and oscillated in a 37°C water bath. The solution was replaced by distilled water at different time points. The collected fluid checked for its absorbance at 590 nm. We then calculated the amount of cumulative release according to the standard curve.

Cell lines and the uptake of Abc-GC-gemcitabine nanoparticles in cells

Human pancreatic cancer cell SW1990 cells was purchased from Shanghai Institutes for Biological Sciences (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco) containing 10% fetal bovine serum (Sigma Aldrich, St. Louis, MO, USA) at 37°C with 5% carbon dioxide incubator.

Fluorescence intensity was used to reflect the uptake of Abc-GC-gemcitabine nanoparticles in cells. Cells were plated into 6-well plates at an initial concentration of 1×10^3 cells per well and treated with GC-gemcitabine microspheres solution (GC group) or Abc-GC-gemcitabine microspheres solution (Abc group). Cells were collected after 6 hours and 12 hours and the fluorescence intensity observed by fluorescence microscope.

Cell proliferation detection

The cell proliferation of SW1990 was assessed using a cell counting kit-8 (CCK-8) assay (Beyotime, Nantong, China) based on the manufacturer's instructions. Briefly, cells were seeded into 96-well plates at a density of 7,000 cells/well and incubated for 24 hours. Cells were then treated with GC-gemcitabine microspheres solution (GC group) or Abc-GC-gemcitabine microspheres solution (Abc group) for 24 hours, 48 hours, or 72 hours. On each monitored day, 10 μ L of CCK-8 solution was added to each well and cells were further incubated at 37°C for 2 hours, absorbance was measured at 450 nm using an electroluminescence immunosorbent assay reader (Thermo Scientific, MA, USA).

Colony formation assay

Anchorage-independent growth was also assessed by colony formation ability [20]. Cells were seeded at 1×10^3 cells/well in 6-well plates and treated with GC-gemcitabine microspheres solution (GC group) or Abc-GC-gemcitabine microspheres solution (Abc group). After 14 days, the adherent cells were washed with PBS twice, fixed with 4% paraformaldehyde, and stained with 0.1% crystal violet (Sigma Aldrich, St. Louis, MO, USA). The total number of colonies (≥ 50 cells/colony) was counted.

Cell migration assay

The migration ability was determined using wound-healing assay. Pancreatic cancer cells were plated into 12-well plates, when reached approximately 90% confluence, a 200- μ L pipette tip was used to scratch the cells layer. After being washed with PBS three times, GC-gemcitabine microspheres solution (GC group) or Abc-GC-gemcitabine microspheres solution (Abc group) were added to each group and the cells were continue to be incubated in medium containing 2% FBS for additional 48 hours. Digital camera system (Olympus Corp., Tokyo, Japan) was used to acquire images of the scratches of the cells at magnification of 200 \times .

Transwell assay

The invasion ability of SW1990 cells were assessed by Boyden chambers (Corning, Corning, NY, USA). After 48 hours treated with GC-gemcitabine microspheres solution (GC group) or Abc-GC-gemcitabine microspheres solution (Abc group), cells were harvested with serum-free DMEM media and 150 μ L of cell suspension (approximate 2×10^4 cells) was placed in the upper chamber (Corning, New York, NY, USA) pre-coated with Matrigel (Corning, NY, USA). Meanwhile, the lower chamber was added with 600 μ L DMEM medium supplied with 10% FBS. After incubated for additional 12 hours, the cells attached to the upper surface of the inserts were softly scraped off, and cells that migrated to the lower side were fixed with 4% paraformaldehyde and stained with crystal violet. After being washed with cold PBS three times, cells from five randomly selected visual fields were counted under an immunofluorescence microscope (100 \times magnification) for further quantification of cells.

Western blotting

Treated cells were harvested and lysed in a mixture of the protease and phosphatase inhibitor. We collected the supernatant fraction and measured the protein concentration with bicinchoninic acid protein (BCA assay kit, Beyotime, China). An aliquot of 20 μ g of denatured protein from each sample was subjected to 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were blocked for two hours with 5% non-fat milk in TBST, followed by incubation at 4°C overnight with antibodies (1: 1,000 dilution). After been incubated with secondary antibody of rabbit horseradish peroxidase-conjugated anti-goat IgG (1: 2,000 dilution; Cell Signaling Technology, Boston, MA, USA) for one hour at ambient temperature. The Western blots were then incubated with enhanced chemiluminescence solution for one minute. Final signals were detected by the ChemiDoc XRS+ Chemiluminescence imaging system (Bio-Rad, Hercules, CA, USA) and measured by ImageJ software (Bio-Rad, Inc., Hercules, CA, USA).

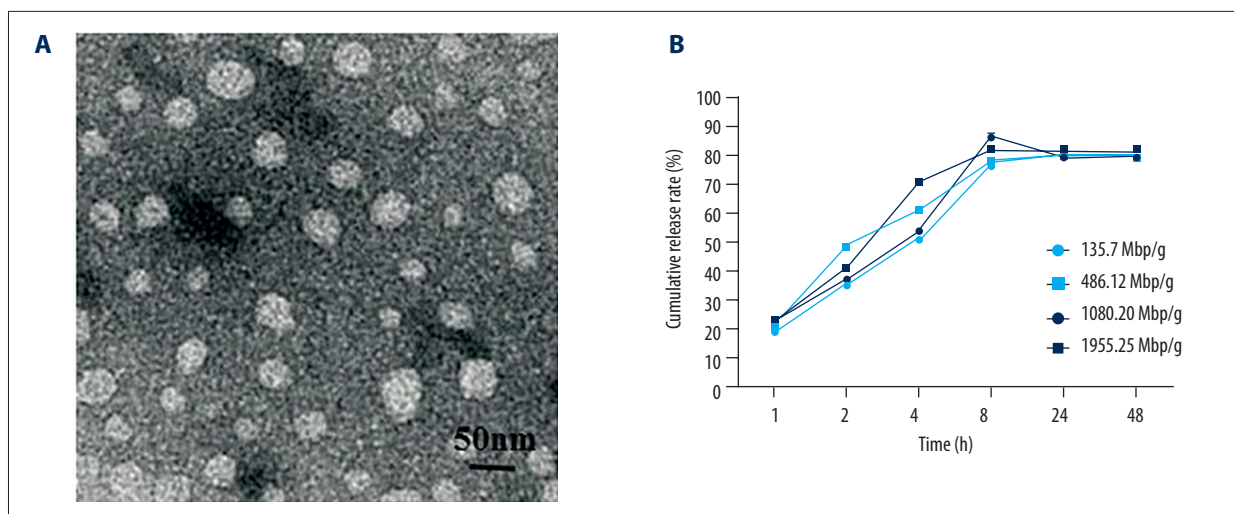


Figure 1. TEM images of Abc-GC-gemcitabine nanoparticles and cumulative release curve of nanobioconjugates. **(A)** TEM images of Abc-GC-gemcitabine nanoparticles revealed that spherical appearance of nanoparticles was neat, the size differences were slight and the dispersity was good. **(B)** The cumulative release curve showed that different drug loadings released into balance after 8–24 hour. The lower amount of drug loadings, the lighter release effect, which balanced for longer time (24 hours) and the role of sustained release was more apparently.

Statistical analysis

Statistical analysis was conducted using SPSS software version 18.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5 (La Jolla, CA, USA). One-way analysis of variance (ANOVA) and Tukey’s post-hoc analysis were used to compare between multiple experimental groups. Student *t*-test was performed for the comparisons between two different groups results were reported at least three independent experiments. A $p < 0.05$ was considered to be a statistically significant difference.

Results

Electron microscope images, encapsulation rate, drug loadings and cumulative release curve of nanobioconjugates

The nanobioconjugates were synthesized for pancreatic cancer treatment as described previously. As shown in Figure 1A, images of Abc-GC-gemcitabine nanoparticles from the transmission electron microscope (TEM) revealed that spherical appearance of nanoparticles was neat, the size differences slight and the dispersity was good. Meanwhile, according to drug loadings and the encapsulation rate meter calculate formula, the encapsulation rate of nanobioconjugates was 91.63%, the drug loadings was 9.97%. In addition, the cumulative release curve showed that different drug loadings released into a balance after 8–24 hours and the cumulative release rate was $86.5 \pm 1.1\%$. The lower amount of drug loadings, the lighter

release effect which balanced for longer time (24 hours), and the role of sustained release was more apparently (Figure 1B).

The uptake of nanobioconjugates in SW1990 cells

As shown in Figure 2A, the uptake of Abc-GC-gemcitabine microspheres for SW1990 cells in the Abc group increased gradually with the extension of time, stronger than the uptake of GC-gemcitabine microspheres at the same point in the GC group. Furthermore, we analyzed the average optical density and the result revealed that the average amount of nanoparticles for SW1990 cells in the Abc group was significantly stronger than the GC group at the same time ($p < 0.01$, Figure 2B).

Nanobioconjugates reduces SW1990 cells proliferation and colony formation

SW1990 cell proliferation was decreased after treatment with nanobioconjugates (Figure 3A). Cell proliferation in the Abc group was inhibited more obviously compared to the GC group ($p < 0.01$). Moreover, colony formation ability of both groups were significantly inhibited by nanobioconjugates ($p < 0.01$, Figure 3B, 3C), and the difference in colony amount between the Abc group and the GC group was obviously ($p < 0.01$). Additionally, Western blot was used to examine increased protein expression levels of PCNA and Ki-67 in SW1990 cells which were also decreased by both nanobioconjugates (Figure 3D).

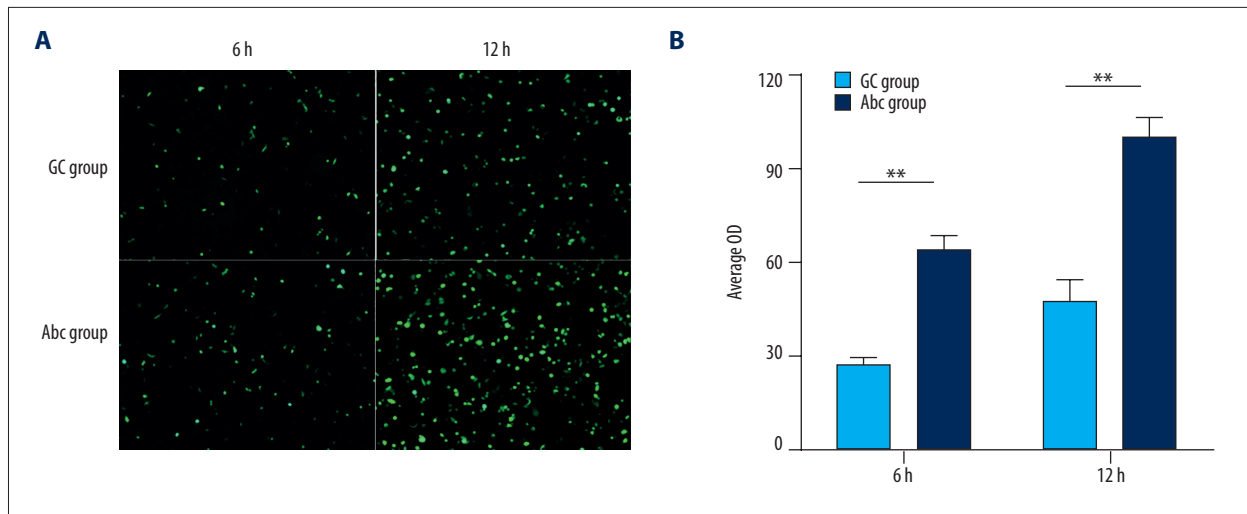


Figure 2. The uptake of nanobioconjugates in SW1990 cells. **(A)** The uptake of nanobioconjugates in SW1990 cells. The uptake of Abc-GC-gemcitabine microspheres for SW1990 cells in the Abc group increased gradually with the extension of time, stronger than the uptake of GC-gemcitabine microspheres at the same point in the GC group. **(B)** The average optical density of nanobioconjugates in SW1990 cells. The result revealed that the average amount of nanoparticles for SW1990 cells in the Abc group was significantly stronger than the GC group at the same time. ** $p < 0.01$.

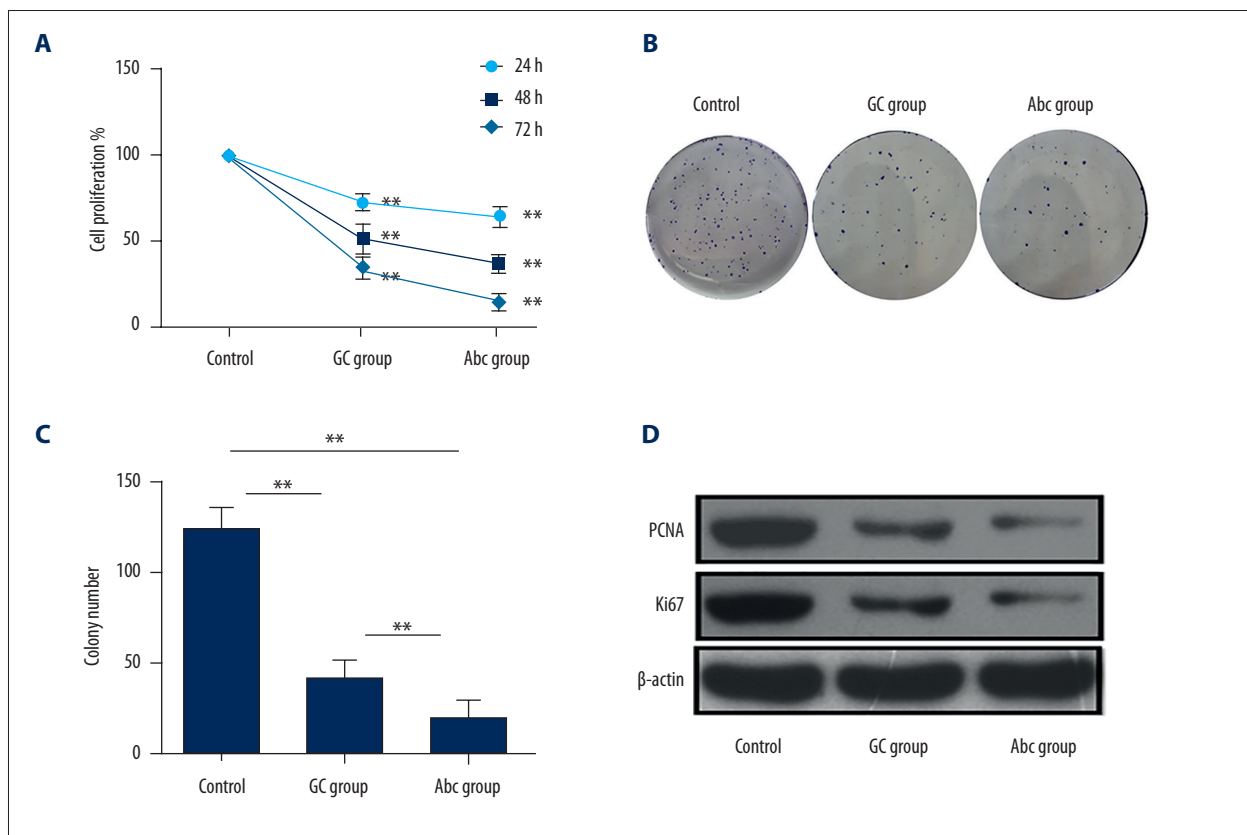


Figure 3. Nanobioconjugates reduces SW1990 cells proliferation and colony formation. **(A)** SW1990 cell proliferation was reduced after treatment with nanobioconjugates. **(B, C)** Colony formation of both groups were inhibited by nanobioconjugates, and the difference in colony amount between the Abc group and the GC group was significant. **(D)** Western blot examined that increased protein expression levels of PCNA and Ki-67 in SW1990 cells were decreased by both nanobioconjugates. ** $p < 0.01$.

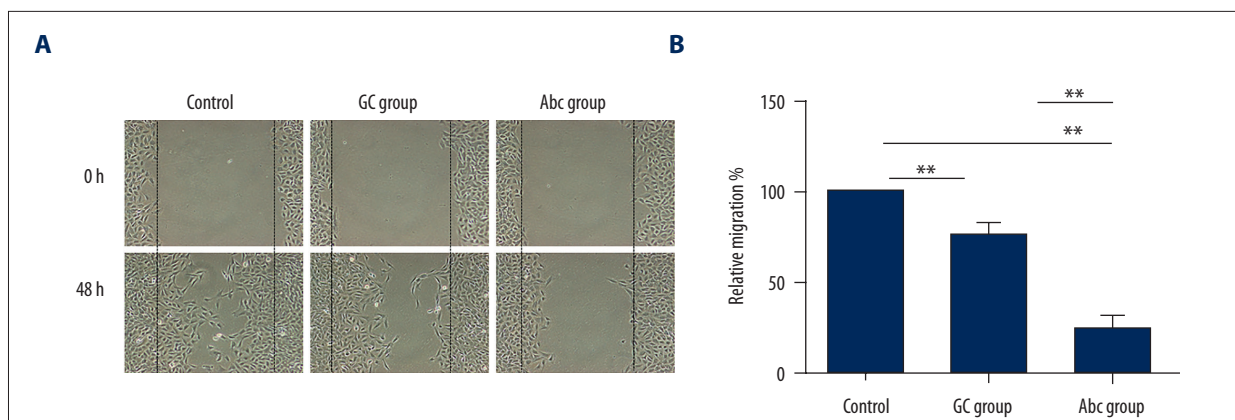


Figure 4. Nanobioconjugates inhibits the migration of SW1990 cells. **(A, B)** Nanobioconjugates inhibit the migration of SW1990 cells. Significant inhibitory effects on the migration were observed in SW1990 cells treated with GC-gemcitabine microspheres solution (GC group) or Abc-GC-gemcitabine microspheres solution (Abc group) for 48 hours. ** $p < 0.01$.

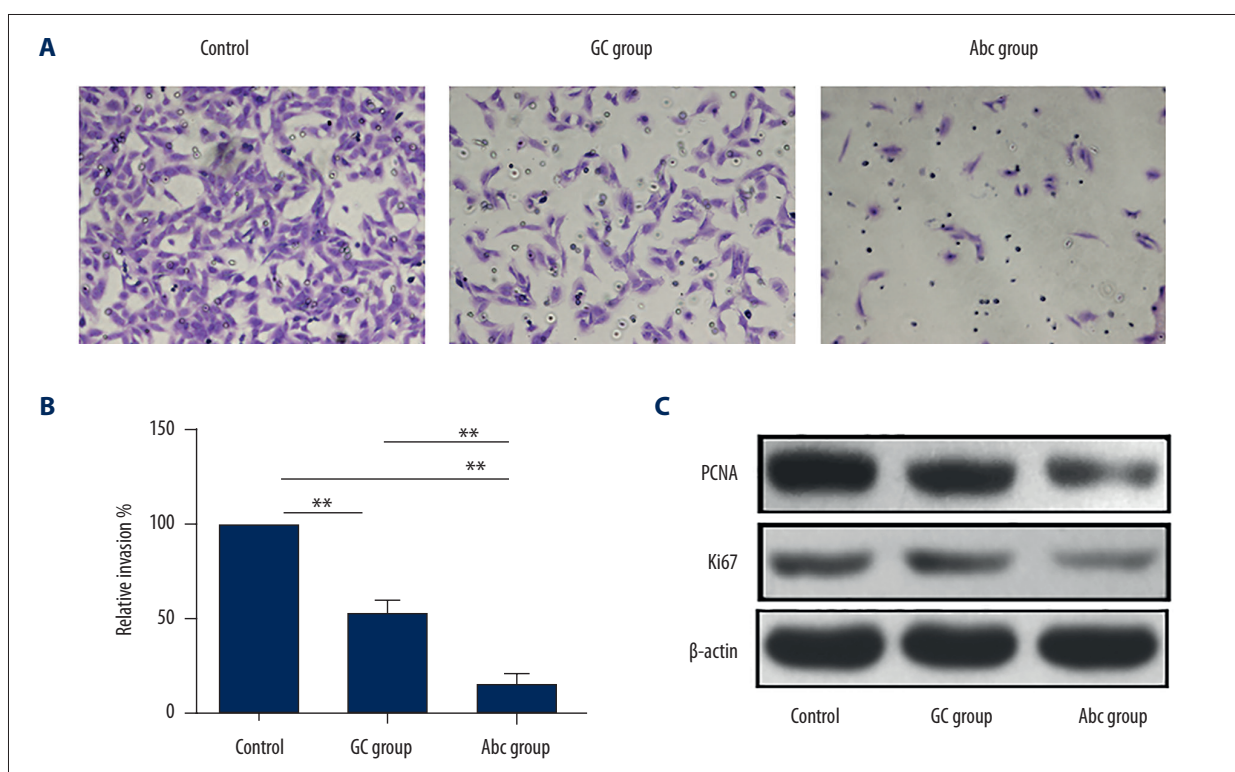


Figure 5. Nanobioconjugates inhibits the invasion of SW1990 cells. **(A, B)** Nanobioconjugates inhibit the invasion of SW1990 cells. **(C)** The protein expression levels of MMP9 and MMP2 in SW1990 cells were decreased dramatically after incubated with GC-gemcitabine nanoparticles or AbC-GC-gemcitabine nanoparticles. ** $p < 0.01$.

Nanobioconjugates inhibits the migration of SW1990 cells

Treated with GC-gemcitabine microspheres solution (GC group) or Abc-GC-gemcitabine microspheres solution (Abc group) significantly inhibited the migration ability of SW1990 cells compared to the control ($p < 0.01$, Figure 4A, 4B). Moreover, the Abc group decreased SW1990 cell migration to a dramatically greater extent than the GC group.

Nanobioconjugates inhibits the invasion of SW1990 cells

Similar to migration, nanobioconjugates treatment resulted in strong invasion inhibitory of SW1990 cells ($p < 0.01$, Figure 5A, 5B). Moreover, the protein expression levels of MMP9 and MMP2 in SW1990 cells were also decreased dramatically after incubated with GC-gemcitabine nanoparticles or AbC-GC-gemcitabine nanoparticles (Figures 5C).

Discussion

In our study, we synthesized Abc-GC-gemcitabine nanoparticles successfully with the encapsulation rate of nanobioconjugates was 91.63% and the drug loadings was 9.97%. When applied to treat human pancreatic cancer cells SW1990, both GC-gemcitabine microspheres solution (GC group) and Abc-GC-gemcitabine microspheres solution (Abc group) expressed significant inhibited action in SW1990 cells compared with the control. Wherein the anti-tumor action for Abc-GC-gemcitabine microspheres was significantly superior compared with GC-gemcitabine microspheres.

Pancreatic cancer has the highest mortality rate among gastrointestinal tumors [21,22]. It remains a serious health problem, with a 5-year survival rate for all stages at <5% [2]. Most patients were diagnosed at an advanced stage with poor prognosis. Since the vast majority of pancreatic cancer patients prefer to chemotherapy, novel and efficiently chemotherapy drugs are urgently needed [23,24]. According to the recommendations of the latest version of National Comprehensive Cancer Network guideline, gemcitabine was regarded as the standard chemotherapy for locally advanced or metastatic pancreatic cancer [25,26]. Many previous studies tried to combine gemcitabine with many cytotoxic or targeted agents to improve the poor prognosis of advanced pancreatic cancer. However, they all failed to obtain satisfactory results [7,27,28].

The double antibodies targeting gemcitabine nanoparticle drug synthesized in our research targeted the surface of pancreatic cancer cells by anti-EGFR antibody, and killed cancers after being taken up in cells. It is the specificity of the combination that makes the nanobioconjugates concentrate in the

tumor target area. As a result, drug concentration in the tumor target area improved, the chemotherapy drug usage and the side-effects also reduced simultaneously [29,30]. In addition, gemcitabine released from the nanodrug carrier in a controlled release manner can ensure efficient long-term therapeutic effect.

Human physiological pH is 7.35–7.45 and chitosan dissolves only in pH <6.5 solution, so we choose chitosan as the matrix for nanobioconjugates, which dissolves in a wider pH soluble solution and has good dispersion and stability. In addition, cell membranes carry a negative charge, so positively charged chitosan not only has good cell membrane penetrability, is easily dissolved in the plasma enzymatic degradation, but also has good biocompatibility with cells.

Conclusions

In summary, Abc-GC-gemcitabine nanoparticles have been successfully developed and the nanoparticles have demonstrated targeted aggregating distribution. Meanwhile, nanobioconjugates could reduce pancreatic cancer cell proliferation and colony formation, and inhibit the migration and invasion of SW1990 cells. Therefore it might be assumed that Abc-GC-gemcitabine nanoparticles can be a potential drug for the clinical treatment of pancreatic cancer in the future. Of course, further studies are needed to verify these results and should involve animal trials and prospective and clinical studies.

Conflict of interest

The authors have no conflicts of interest to declare.

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