Efficient pre-treatment for pancreatic cancer using chloroquine?loaded nanoparticles targeting pancreatic stellate cells

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Efficient pre-treatment for pancreatic cancer using chloroquine-loaded nanoparticles targeting pancreatic stellate cells

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Abstract. Pancreatic stellate cells (PSCs) play a key role in desmoplastic stroma, which is a characteristic of pancreatic ductal adenocarcinoma (PDAC), and they also enhance the malignancy of pancreatic cancer cells. Our previous study reported chloroquine's mitigating effects on PSC activation; however, the drug is known to induce adverse effects in clinical practice. The present study aimed to reduce chloroquine doses and develop a useful pre-treatment that targets PSCs using nanoparticles. Poly lactic-co-glycolic acid (PLGA) nanoparticles were used as carriers and loaded with indocyanine green (Nano-ICG) or chloroquine (Nano-CQ). Tumor accumulation of Nano-ICG was evaluated using an *in vivo* imaging system. The effects of chloroquine, Nano-CQ and/or chemotherapy drug gemcitabine were investigated in an orthotopic xenograft mouse model. Nano-ICG selectively accumulated in pancreatic tumors and persisted therein for over 7 days after administration. Additionally, Nano-ICG accumulated in the peritoneal metastasized regions, but not in the liver, kidney and normal pancreatic tissues. Nano-CQ reduced the density of activated PSCs at lower chloroquine doses and significantly restrained tumor progression in combination with gemcitabine. In conclusion, the PLGA nanosystem successfully delivered the drug to pancreatic tumors. Nano-CQ efficiently reduced PSC activation and may be a promising novel pre-treatment strategy for PDAC.

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Abbreviations: αSMA, α-smooth muscle actin; DDS, drug delivery system; DMEM, Dulbecco's modified Eagle's medium; EPR effect, enhanced permeability and retention effect; imPSC, immortalized pancreatic stellate cell; IVIS, *in vivo* imaging system; Nano-CQ, nanoparticles loaded with chloroquine; Nano-ICG, nanoparticles loaded with indocyanine green; PBS, phosphate-buffered saline; PCC, pancreatic cancer cell; PDAC, pancreatic ductal adenocarcinoma; PDAC, pancreatic ductal adenocarcinoma; PLGA, poly lactic-co-glycolic acid; PSC, pancreatic stellate cell

Key words: chloroquine, combination therapy, desmoplasia, nanoparticles, orthotopic xenograft, pancreatic cancer, pancreatic ductal adenocarcinoma, pre-treatment, stroma

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most refractory cancers in humans, with an overall 5-year survival rate of only 9% (1,2). PDAC has an extremely poor prognosis that has not improved much in the last few decades (3,4). Most PDAC cases are diagnosed at an advanced stage, and patients with unresectable PDAC generally receive systemic chemotherapy. Most of the operative cases also need adjuvant chemotherapy to prevent tumor recurrence of cancer. However, the beneficial effects of standard regimens of chemotherapy are not sufficient, giving rise to an urgent requirement of a novel treatment strategy to improve the prognosis of PDAC.

Pancreatic ductal adenocarcinoma is generally characterized by desmoplasia, which consists of an extracellular matrix and stromal cells, including pancreatic stellate cells (PSCs). Desmoplasia is considered one of the main reasons for the resistance of pancreatic cancer to chemotherapy (5,6). Furthermore, activated PSCs in the tumor microenvironment enhance the malignancy of pancreatic cancer cells (PCCs), namely their ability to invade and metastatize (7,8). Therefore, modulating the activation of PSCs is a promising therapeutic strategy for improving PDAC prognosis. We have previously reported the suppression of PSC activation by inhibition of PSC autophagy using chloroquine, a lysosomal inhibitor, and mitigation of tumor progression by chloroquine in a murine PDAC model (8). There are also other reports demonstrating the use of chloroquine for treating PDAC; some indicating promising results (9-12). Long-term use of chloroquine, however, leads to adverse effects, such as retinopathy and cardiac complications, in the clinical setting (12-14).

In the past few decades, various tumor-specific drug delivery systems (DDSs) using nanotechnology such as polymers and liposomes, have emerged (15). In 1986, nano-sized DDS was advocated, which selectively accumulated in tumor tissues due to its enhanced permeability and retention (EPR) effect (16,17). Research has shown promising effects of nanoparticles, loaded with therapeutic agents, on several types of cancers, although this research remains limited overall (15). Inter-alia, poly lactic-co-glycolic acid (PLGA) nanoparticle is an outstanding DDS carrier, which has a high safety profile, approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA), morphological stability, and excellent biocompatibility and biodegradability (18,19).

In this study, we hypothesized that the treatment of PSCs with DDS nanoparticles loaded with chloroquine could suppress PSC activation efficiently and regulate desmoplasia. We developed a novel nano-drug of chloroquine-loaded PLGA nanoparticles (Nano-CQ), aiming to enhance the effect of anti-cancer drugs, like gemcitabine, by anti-stromal pre-treatment with Nano-CQ in an *in vivo* murine PDAC model. To the best of our knowledge, the effect of chloroquine-loaded PLGA nanoparticles targeting PSCs on PDAC *in vivo* as a pre-treatment for chemotherapy has not been previously evaluated.

Materials and methods

Cells. We extracted human PSCs from fresh pancreatic cancer surgical specimens using the outgrowth method and immortalized them as described in our previous reports (20,21). The pancreatic cancer cell line SUIT-2 was purchased from Japan Health Science Research Resources Bank (Osaka, Japan). Both PSCs and SUIT-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37˚C and 10% CO_2, as described previously (22). Cells were confirmed to be free of mycoplasma and used within eight passages for each experiment.

Treatment agents. Indocyanine green was purchased from Tokyo Chemical Industry (#I0535). Chloroquine phosphate was purchased from Sigma-Aldrich (#PHR1258). Gemcitabine was purchased from Eli Lilly, Japan [Gemzar Injection (4224403D1030)]. Medetomidine was purchased from Kyoritsu Seiyaku. Midazolam was purchased from Sandoz. Butorphanol was purchased from Meiji Seika Pharma. We consigned the manufacturing of the PLGA nanoparticles, namely the indocyanine green-loaded nanoparticles (Nano-ICG) and chloroquine-loaded nanoparticles (Nano-CQ), to Sentan Iryou Kaihatsu. PLGA was used as a matrix for the nanoparticles, and polyvinyl alcohol was used as a dispersing agent. The particle size was analyzed by light scattering method, using the Nanotrac Wave-EX150 system (Microtrac BEL Corp.); the median diameters (D50) of Nano-ICG and Nano-CQ were 210 and 205 nm, respectively. These nanoparticles contained 4% indocyanine green (wt/wt) and 1.5% chloroquine (wt/wt), respectively. All the agents were dissolved in phosphate-buffered saline (PBS).

Mice. Seven-week-old BALB/c AJcl nu/nu female mice were purchased from Clea. After one week of acclimation, the mice received immortalized PSCs ($5x10^5$) with SUIT-2 cells ($5x10^5$) or luciferase-expressing SUIT-2 cells ($5x10^5$) suspended in 50 μl DMEM. Cells were orthotopically transplanted into the pancreatic tail under general anesthesia, using combination anesthetic (0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol) administered by intraperitoneal injection (23). The median body weight of mice was 21.63 g (ranges, 19.51-23.79) at the time of implantation. They were fed *ad libitum* and were kept under a 12-h light/12-h dark cycle. The mouse room was kept at a temperature of 20-26˚C and a humidity of 40-70%. All mice were euthanized by dislocating cervical vertebra under general anesthesia at the end of experiments or if a humane endpoint was reached; defined as a loss of >15% of body weight, a tumor volume >1.2 cm^3, severe ascites, vomiting, or inability to ambulate or rise for food and water. However, no animals reached these humane endpoints.

Tumor accumulation assay. Tumor implantation and dissemination were verified by measuring luciferin emission using the *in vivo* imaging system (IVIS) Spectrum (Caliper Life Sciences), after injecting 150 mg D-luciferin (#LK10000; Oz Biosciences) into the intraperitoneal cavity of mice. After verifying tumor formation, either Nano-ICG (0.2 mg indocyanine green per mouse, n=4) or free indocyanine green (0.2 mg per mouse, n=4) was injected into a tail vein. Indocyanine green fluorescence was measured continuously using the IVIS Spectrum (excitation: 745 nm, emission: 840 nm). Luciferin emission and indocyanine green fluorescence were quantified using Living Image software, version 4.1 (Summit Pharmaceuticals International Corporation).

Immunohistochemistry. Formalin-fixed, paraffin embedded tumor tissues were sliced into 4-μm-thick sections and endogenous peroxidase activity was blocked with methanol containing 0.3% hydrogen peroxidase for 30 min at room temperature. Antigen retrieval was performed by boiling in a microwave oven (citrate buffer, pH 6.0). Afterwards, the tissues were incubated with mouse anti-α-smooth muscle actin (αSMA) antibody (1:100, #M0851; Dako) overnight at 4˚C and stained with EnVision+System-HRP Labeled Polymer Anti-Mouse (#K4001; Dako). Activated PSCs were identified based on their spindle-like shape and αSMA-positive staining. The αSMA-positive area was measured using analysis application Hybrid cell count (BZ-H3C, Keyence).

Cellular uptake assay. SUIT-2 cells ($5x10^5$/well) or immortalized pancreatic stellate cells (imPSCs) ($5x10^5$/well) were cultured in a 6-well plate. After 24 h of incubation at 37˚C and 10% CO_2, cells were treated with Nano-ICG (10 μM indocyanine green) or free indocyanine green (10 μM) for 1 h and washed with PBS. The intracellular indocyanine green fluorescence was observed under BZ-X700 (Keyence) fluorescent microscope. Nuclei were counterstained using Hoechst 33342 (#H342; Dojindo). Each experiment was conducted in triplicate wells and repeated twice.

In vivo treatment experiment. We conducted the treatment experiments in seven different groups using the SUIT-2 cells and imPSCs co-transplanted model: chloroquine group (n=3), low-dose chloroquine group (n=3), Nano-CQ group (n=3),

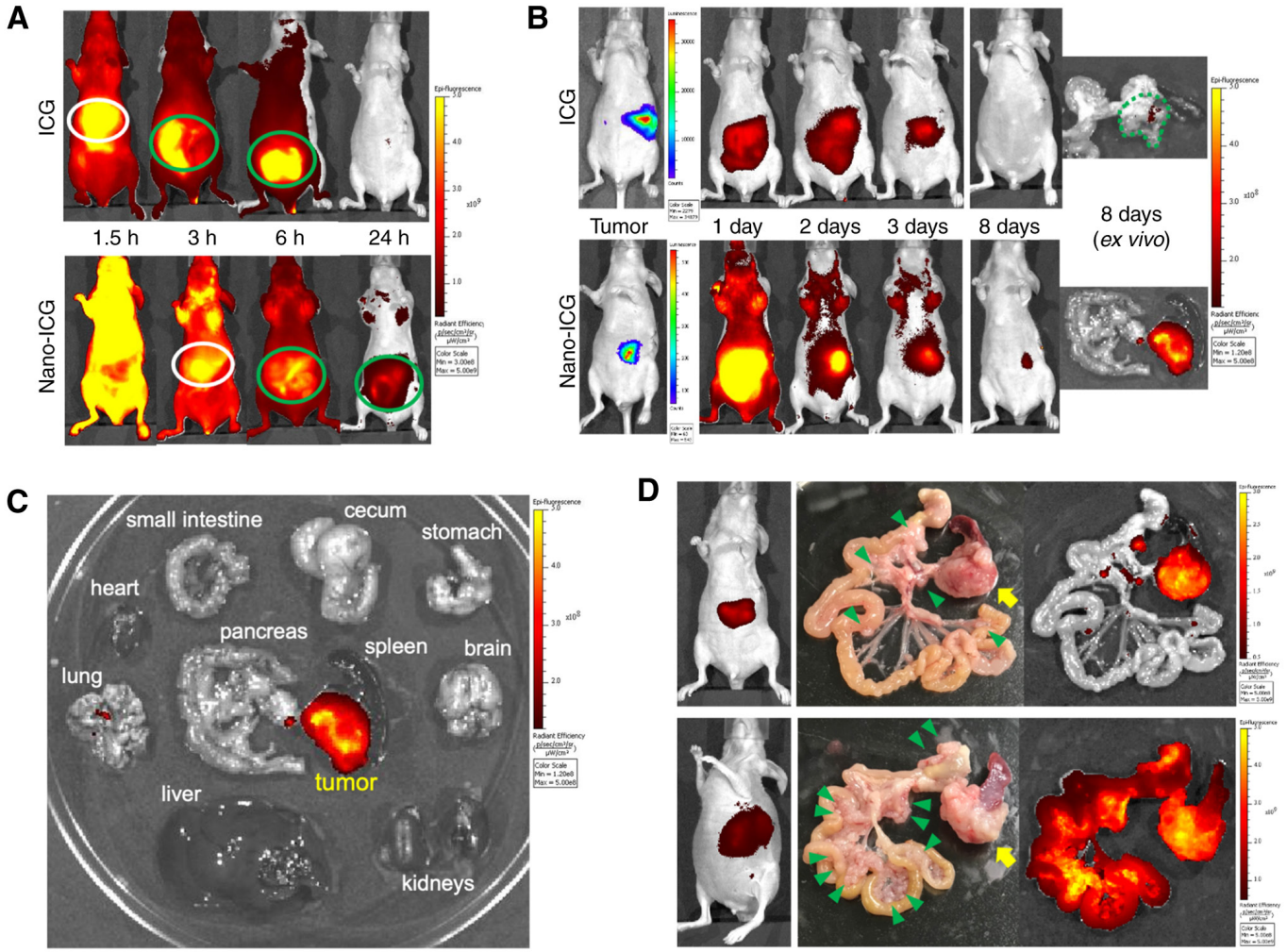

Figure 1. Nano-ICG shows accelerated retentivity in the blood and tumor-selective accumulation. Representative images of the IVIS in mice orthotopically co-implanted with luciferase-expressing SUIT-2 cells and imPSCs after intravenous administration of free ICG (top row) or Nano-ICG (bottom row). (A) Comparison of retentivity between free ICG and Nano-ICG. Nano-ICG showed accelerated retentivity in the blood and delayed timing of uptake into the liver (white circles) and discharge into the intestine (green circles) compared with free ICG. (B) Comparison of tumor accumulation between Nano-ICG and free ICG. Nano-ICG indicated enhanced accumulation in murine pancreatic tumor tissue and persisted there markedly longer compared with free ICG. (C) Accumulation of Nano-ICG in major organs 8 days after administration. Nano-ICG selectively accumulated in the tumor tissues. (D) Accumulation of Nano-ICG in the metastasized regions. Nano-ICG accumulated in disseminated nodules (green arrowheads) as well as primary pancreatic tumor tissue (yellow arrows). ICG, indocyanine green; imPSC, immortalized pancreatic stellate cell; IVIS, *in vivo* imaging system.

gemcitabine group (n=3), gemcitabine + chloroquine group (n=3), gemcitabine + Nano-CQ group (n=4), and control (vehicle) group (n=6). The treatment agents were administered according to the following schedule: cells were implanted on day 0; chloroquine (50 mg/kg) was administered on days 7, 8, 9, 14, 15, 16, 21, 22 and 23; low-dose chloroquine (30 mg/kg), Nano-CQ (30 mg/kg chloroquine), and vehicle were administered on days 7, 14 and 21; gemcitabine (40 mg/kg) was administered on days 10, 17 and 24. All mice were sacrificed on day 28, with all orthotopic tumors were resected and measured. Tumor volume was calculated using the following formula: $\pi/6$ x L x W x H, where L is the largest tumor diameter, W is the smallest diameter, and H is the height. The maximum tumor volume was ~0.8 cm^3.

Statistical analysis. Results are presented as the mean ± standard error. The effects of Nano-CQ on tumor volume, tumor weight and αSMA expression were analyzed using ANOVA followed by the Tukey's test. Statistical significance was defined as $P<0.05$. All statistical analyses were performed using JMP 14 software (SAS Institute).

Results

PLGA nanoparticle acts as a drug delivery system in pancreatic tumor. The ability of PLGA nanoparticles to deliver drugs to the murine pancreatic tumor was evaluated using Nano-ICG. Nano-ICG or free indocyanine green was injected into a tail vein of the mouse orthotopically co-implanted with PCCs and PSCs. *In vivo*, indocyanine green is metabolized in the liver and subsequently secreted into the bile (24,25). On short-term observation, free indocyanine green was taken into the liver and secreted into the bile; thereafter, it disappeared from the systemic circulation quickly and was discharged through the intestine. In contrast, Nano-ICG needed substantially more time for liver uptake after administration (Fig. 1A).

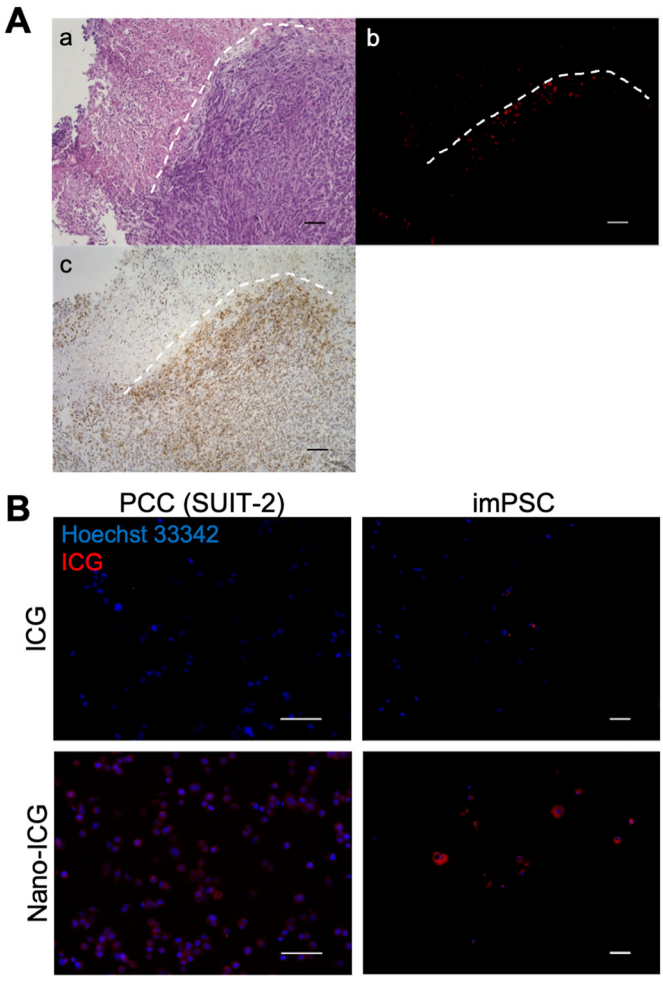

Figure 2. Nano-ICG preferentially accumulates in PSCs. (A) Representative photomicrographs of orthotopic tumor stained with (a) HE, (b) fluorescence for ICG and (c) αSMA immunostaining. Nano-ICG was observed in the αSMA-positive area. The white, short, dashed lines show the contour of the viable tumor. Magnification, x100; scale bars, 100 μm. (B) Representative photomicrographs of PCCs (SUIT-2) and imPSCs washed at 1 h after addition of free ICG or Nano-ICG. Nano-ICG showed more intracellular uptake compared with free ICG, and PSCs showed stronger intracellular fluorescence of Nano-ICG compared with PCCs. Original magnification, x200 (left panels) or x100 (right panels); scale bars, 100 μm. HE, hematoxylin eosin; αSMA, α-smooth muscle actin; PCC, pancreatic cancer cell; ICG, indocyanine green; PSC, pancreatic stellate cells; im, immortalized.

These results indicate the enhanced retentivity of this PLGA nanocarrier in the blood.

Long-term observations revealed the accumulation and retention of Nano-ICG particles for 8 days in the pancreatic tumor. On the other hand, no obvious accumulation of free indocyanine green was observed in the tumors 8 days after administration (Fig. 1B). These findings suggest that the EPR effect of this nanosystem is sufficient *in vivo* applications. Furthermore, there was no obvious accumulation of Nano-ICG in other major organs, including normal pancreatic tissue (Fig. 1C). Nano-ICG accumulated in disseminated nodules as well as in the primary pancreatic tumor, regardless of their number or size (Fig. 1D). Based on these findings, it is clear that this DDS can be used to treat not only localized PDACs but also metastatic ones.

Histologically, Nano-ICG accumulated in the αSMA-positive area (Fig. 2A). *In vitro* analysis showed that PSCs, rather than PCCs, had higher fluorescent intensity for Nano-ICG and Nano-ICG showed much improved intracellular uptake than free indocyanine green in both PCCs and PSCs (Fig. 2B). These findings suggest that the uptake of PLGA nanocarrier is higher in PSCs than PCCs.

Nano-CQ efficiently reduces the density of activated PSCs and enhanced the antitumor effect of gemcitabine. To assess the antitumor effects of chloroquine-loaded PLGA and its potential effects on PSC activation, we used a single administration of Nano-CQ (total chloroquine dose: 90 mg/kg), free chloroquine (total dose: 90 mg/kg or 450 mg/kg), or combination therapy with the anti-cancer drug gemcitabine, in the orthotopic murine PDAC model (Fig. 3A). The treatment started 7 days post-implantation and the antitumor effect was assessed by measuring the tumor volume and weight on day 28. The gemcitabine, gemcitabine + chloroquine, and gemcitabine + Nano-CQ groups showed the antitumor effect compared with the control (vehicle) group and the latter two groups seemed to show the more effect than the former, although no statistical significance was detected (Fig. 3B-D); indicating that Nano-CQ successfully enhanced the effect of gemcitabine at the lower chloroquine dose.

The effect of Nano-CQ on PSC activation was assessed using immunohistochemistry of αSMA. The αSMA-positive area in the tumor was significantly decreased in the Nano-CQ ($P=0.001$) and chloroquine ($P<0.001$) groups, compared to the control group (Fig. 3E and F). Similar results were observed in the combination therapy groups (Fig. 3E and F). Of note, no inhibitory effect on PSC activation was observed in the low-dose chloroquine group, which had the same chloroquine dosage as the Nano-CQ group (Fig. 3E and F).

Discussion

In this study, Nano-ICG showed great ability to selectively accumulate in pancreatic tumor tissues and to remain there for much longer than free indocyanine green, in a murine PDAC model. Our findings suggest that this PLGA nanoparticle-DDS could be useful for the treatment of PDAC. Furthermore, Nano-CQ successfully enhanced the antitumor effect of gemcitabine by reducing the density of activated PSCs in the tumor tissue.

Consistent with previous reports, free indocyanine green showed rapid uptake by the liver and quick disappearance from the circulation in mice (26,27). In contrast, Nano-ICG uptake and retention by the liver was much higher and longer than free indocyanine green. These findings indicate that this nanocarrier has enhanced retentivity in the blood and it avoids the reticuloendothelial system and opsonization due to the presence of a hydrophilic surface layer of polyvinyl alcohol (18,28). Nano-ICG showed highly selective accumulation in the tumor of the orthotopic murine PDAC model without the need for any active targeting, such as tumor-specific antibodies (29,30); this is due to Nano-ICG's EPR effect, suggesting this nanosystem is a useful DDS for the treatment of pancreatic tumor. Furthermore, Nano-ICG showed high accumulation in the disseminated nodules, indicating that this DDS may be beneficial in treating PDAC since more than half of the patients with this cancer have

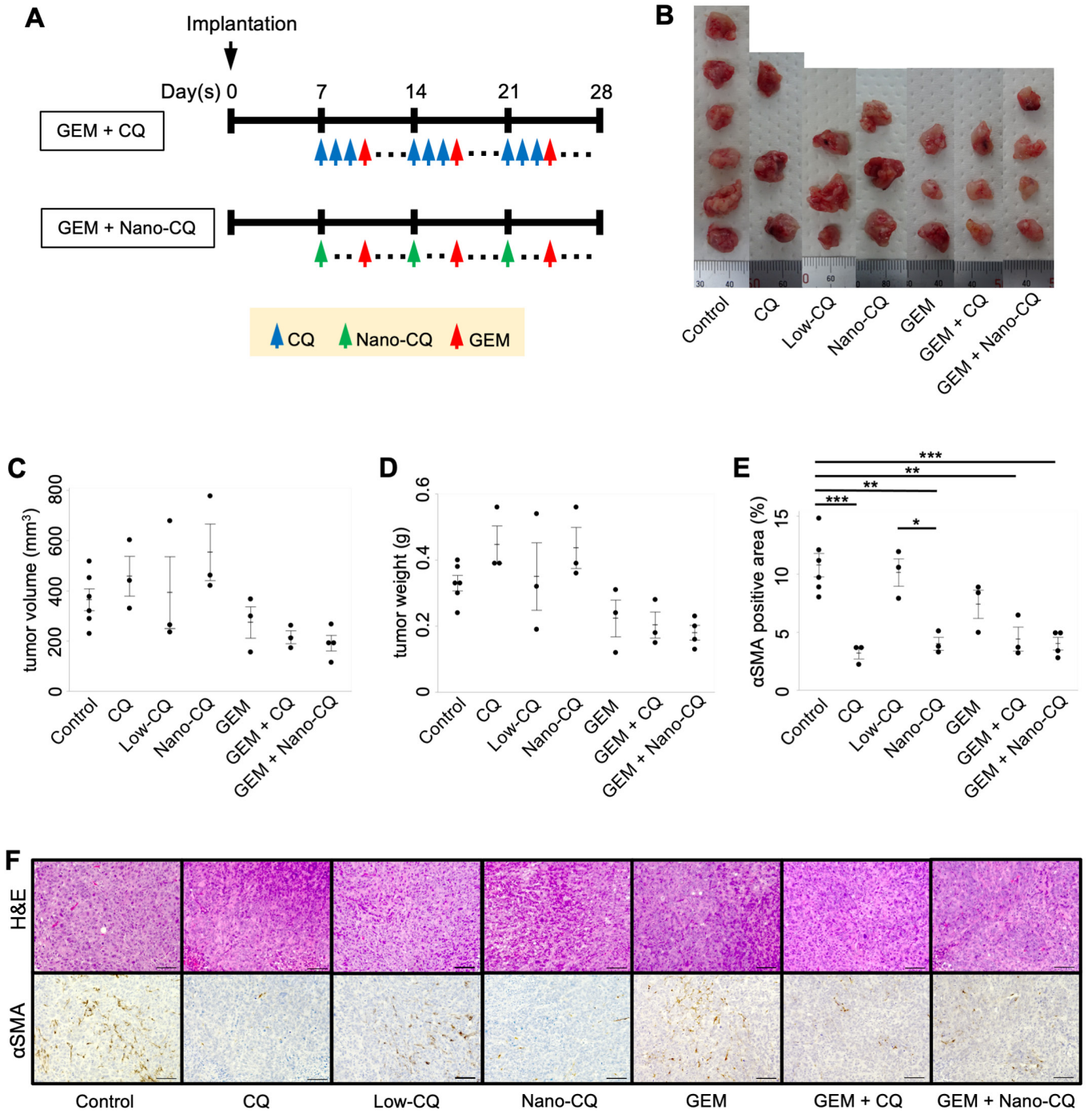

Figure 3. Nano-CQ efficiently reduces the density of activated PSCs and enhances the effect of gemcitabine. (A) Schema of treatment schedules for each group. The mice orthotopically co-implanted with SUIT-2 cells and imPSCs were treated with the single use of Nano-CQ, free chloroquine or the combination of these agents and GEM. (B) Representative images of harvested pancreatic tumors in each group (n=3-6 per group). (C) Volume and (D) weight of tumors were significantly reduced in the Nano-CQ and GEM combination therapy group. (E) αSMA-positive area was significantly decreased in the groups with Nano-CQ or free CQ treatment compared with that of the control group. (F) Representative photomicrographs of orthotopic tumors with HE and αSMA staining (magnification, x200; scale bars, 100 μm). *P<0.05. **P<0.01. ***P<0.001. GEM, gemcitabine; CQ, chloroquine; HE, hematoxylin and eosin.

metastasis at the time of diagnosis (2). In addition, although the effect of this DDS was evaluated in a murine PDAC model in this study, this simple DDS could be applied to the tumors of other organs or even nontumorous diseases, such as inflammatory diseases because it functions by passive targeting (18,19,31).

The distribution of Nano-ICG was mostly observed in the αSMA-positive area, which was located in the peripheral region of the tumor tissues, suggesting higher Nano-ICG uptake by PSCs than PCCs. Indeed, studies have shown that PSCs have a higher uptake capacity for extracellular components and drugs than PCCs (32,33). Altogether, this nanosystem is considered to affect PSCs more strongly than PCCs. Moreover, high interstitial fluid pressure and low perfusion due to poorly developed blood vessels are considered to be the main reasons for inefficient drug delivery to cancer cells in solid tumors (5,6,34-36).

Thus, Nano-ICG was observed in the peripheral region of the tumor tissues.

Recently, chloroquine has demonstrated the ability to interfere with various physiological processes, a property that might be useful in cancer therapy (12,37). We have previously reported on chloroquine's antitumor property, inhibiting cancer-stromal interaction via inhibition of PSC autophagy (8). However, the dose we used in this study was much lower than the dose used in previous studies (8,12). Thus, the single use of Nano-CQ or free chloroquine could not show antitumor effects in the present study.

We have measured αSMA as it is a known marker of activated PSCs (38,39), which play a central role in producing extracellular matrix to form desmoplasia. In this study, we intended to modulate the desmoplastic stroma of PDAC and to make it easier for chemotherapeutics to reach the cancer cells by administrating Nano-CQ as a pre-treatment agent. Thus, we considered that reducing activated PSCs was the most important indicator. A similar reduction in the density of activated PSCs was achieved in the Nano-CQ group, compared to the chloroquine group, in spite of using one-fifth of the chloroquine dose used in the former compared to the latter group. In contrast, low-dose free chloroquine, which had the same chloroquine dosage as in the Nano-CQ group, did not reduce PSC activation. These results suggest that this nanosystem could reduce the dose of chloroquine needed while preserving its effectiveness. Considering the adverse effects of chloroquine, including retinopathy and cardiac complications, which have been well-established in clinical practice (12-14), this DDS could help reduce these risks by its high tumor selectivity and low chloroquine doses needed.

In this study, we aimed to enhance the antitumor effect of gemcitabine via modulation of desmoplasia in PDAC using pre-treatment with Nano-CQ. This combination therapy showed promising results, as intended. Nano-CQ preferentially accumulated in PSCs in the pancreatic tumor, reduced the density of activated PSCs before administration of gemcitabine, and then synergistically enhanced the antitumor effect of gemcitabine successfully. These findings indicate that Nano-CQ shows long-lasting effectiveness, at lower doses, due to its highly selective tumor accumulation, retention, and sustained drug release property. However, according to some reports, the EPR effect of nano drugs needs to be augmented by enhancing tumor blood flow using vasodilators and other drugs for further effectiveness in the clinical setting (40-43). Such augmentation of tumor blood flow may further enhance Nano-CQ accumulation in tumors.

The findings of this study have some limitations. First, we did not conduct a large-scale treatment experiment that could serve as a basis for clinical application. Second, we decided treatment regimens, such as the dose of drugs, based on previous studies. Further research is needed, however, to determine the optimal regimen. Third, the optimization of the size and drug content rate of the PLGA nanoparticles is also needed in the future research.

In conclusion, the nanosystem described in our study, which is based on PLGA nanoparticles, was capable of acting as a DDS for pancreatic tumors, even in those inducing metastasis. Moreover, Nano-CQ pre-treatment efficiently enhanced the antitumor effect of gemcitabine by reducing PSC activation *in vivo*. Nano-CQ showed an adequate effect at lower doses of chloroquine, compared to free chloroquine, indicating that this nanosystem could reduce the dose of chloroquine needed and, consequently, mitigate its adverse effects. Although further large-scale studies and investigations are needed to translate our findings to the clinical practice, including determining the optimal drug concentration in the nanocarrier, dose, and schedule of treatment, Nano-CQ offers a promising pre-treatment strategy for PDAC in combination with gemcitabine.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SM, KN, KO and MN conceived and designed the study. SM performed all experiments and data curation, and drafted the manuscript. SM, AS, WG and NI analyzed and interpretated the curated data. KN and MN supervised the project, and confirm the authenticity of all the raw data. SM, KN, NI and KO revised the manuscript. MN approved the final version of the manuscript for publication. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all the participants before using their pancreatic cancer surgical specimens for the establishment of PSCs, and the study was approved by The Ethics Committee of Kyushu University (approval no. 2019-462; Fukuoka, Japan). All animal experiments were approved by The Ethics Committee of Kyushu University (approval no. A20-248-1; Fukuoka, Japan) and were conducted in accordance with the Japanese laws associated with animal experiments, the institutional regulations of Kyushu University, the Science Council of Japan guidelines for proper conduct of animal experiments (44) and the National Institutes of Health guide for the care and use of laboratory animals (45).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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