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ORIGINAL RESEARCH

The Anti-Tumor Effect of Nab-Paclitaxel Proven by Patient-Derived Organoids

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Background: Nab-paclitaxel has been widely used in treating breast cancer and pancreatic patients for its low toxicity and high efficiency. However, its role in gastric cancer (GC) remains ambiguous. The aim of our study was to test the anti-tumor activity of nab-paclitaxel using GC patient-derived organoids.

Methods: By using the organoid culture system, we describe the establishment of human gastric cancer organoid lines from surgical samples of three patients with gastric cancer. The consistency of these organoids with original cancer tissues was evaluated by histopathological examination. The characteristics of the cancer organoids were tested using immunofluorescence (IF) staining. Using organoids, the anti-tumor efficiencies of nab-paclitaxel, 5-Fu and epirubicin were compared by CCK8 assay and Annexin V-FITC/PI staining.

Results: Three organoids were successfully established and passaged. The morphology of the established GC organoids was consistent with original cancer tissues. The IC50 of nab-paclitaxel was 3.68 μmol/L in hGCO1, 2.41 μmol/L in hGCO2 and 2.91 μmol/L in hGCO3, which was significantly lower than those of 5-FU (72.99 μmol/L in hGCO1, 28.32 μmol/L in hGCO2 and 2.91 μmol/L in hGCO3) and epirubicin (25.85μmol/L in hGCO1, 15.15 μmol/L in hGCO2 and 7.60 μmol/L in hGCO3). When each organoid lines were treated with nab-paclitaxel for increasing period of time, the percentage of the apoptotic cells in each organoid increased accordingly.

Conclusion: Nab-paclitaxel showed strong anti-tumor activity and had the potential to become front-line drug for treating GC patients. Gastric cancer organoid may be a good tool to predict in vivo response to drugs.

Keywords: nab-paclitaxel, organoid, gastric cancer, anti-tumor

Introduction

Gastric cancer (GC) is one of the most prevalent malignancies worldwide, and ranks second in both incidence and mortality rate.[1–3] Recent epidemiological data revealed that most GC patients are locally or distantly advanced when the diagnosis is made.[4] For locally advanced GC patients, curative surgery is still the first choice, after which adjuvant chemotherapy is given to make sure the remnant cancer cells are destroyed. For patients with distantly advanced GC, palliative therapy is the only option. Despite great advances have been made in the area of surgical techniques, it is still very ineffective in advanced GC. In addition, conventionally used drugs are only effective in a fraction of patients. All these facts make the development of more effective therapies an urgent task.

By far, cell lines and patient-derived xenografts (PDX) are the two most commonly used human-derived GC models.[5,6] However, these two models have

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some significant limitations. On one side, cell lines are difficult to establish and cannot accurately simulate the biological behavior of malignant tumors; on the other side, although PDX models can accurately simulate the biological behavior of malignant tumors, they are expensive and inconvenient to establish. These limitations make it hard for them to be commonly used in clinical settings. Additionally, both models are usually derived from advanced-stage tumors and consequently do not fully represent the whole picture of GC. Over the past few years, great advances have been made in the area of organoids culture, which has drew more and more attentions from scientists around the world. Organoids are three-dimensional ex vivo models that can accurately simulate the in vivo conditions. Using organoids models to study the behavior of malignant cells and their interactions with the microenvironment is a hot area of research.[7]

Paclitaxel and docetaxel are two classical microtubule inhibitors that exert their activity by promoting tubulin polymerization and stabilization of microtubules, which results in G2-M phase arrest and mitotic cell death.[8,9] They have been widely adopted in treating breast cancer and pancreatic cancer patients. Despite being quite effective, severe hypersensitivity and other potentially dramatic side effects can be caused by paclitaxel and docetaxel.[10,11] Nab-paclitaxel is an equivalent effective, yet more tolerable substitute for paclitaxel and docetaxel. It is widely adopted in clinical practice, especially in treating breast cancer and pancreatic cancer. Despite a few studies on the application of nab-paclitaxel in GC have been published, the role of nab-paclitaxel in treating GC is still not fully clarified.[12–17]

In the present study, we established and characterized three GC-derived organoids. The anti-tumor activity of nab-paclitaxel and two other conventional chemotherapeutic drugs were compared using these successfully established organoids.

Methods

Gastric Cancer Tissue Processing

This study was approved by the ethical committee of our institution. Informed consents were obtained with each patient. This study is performed in compliance with the Declaration of Helsinki. Tumor tissues were instantly obtained after gastric cancer were removed from the patients. Tumor tissues were kept in DPBS without Ca2+ and Mg2+ supplemented with antibiotics and minced into pieces of 1–3 mm^3 in size. Two random pieces from each specimen were fixed in formalin for histopathological and immunohistochemical analyses and the remaining were processed for the isolation of viable cells. The remaining tissues were minced and then washed with 10 mL AdDF+++ (Advanced DMEM/F12 containing 1x Glutamax, 10 mM HEPES, and antibiotics). The tissues were then digested in 10 mL GC organoid medium containing 1–2 mg/mL collagenase (Sigma, C9407) on an orbital shaker at 37°C for 1–2 h. Then the acquired tissue suspension was sequentially sheared using 10 mL and 5mL plastic and flamed glass Pasteur pipettes. After every shearing the suspension was strained over a 100 μm filter with retained tissue pieces entering a subsequent shearing step with ~10mL AdDF+++ centrifugation at 300 rcf.

GC Organoid Culture

The resuspension was mixed with Matrigel at the ratio of 2: 1. The mixed liquid was added into prewarmed 6-well suspension culture plates with each drop of 40 μL and these plates were then put into an incubator chamber at 37°C for 10 minutes. Upon gelation completed, 1.5 mL IntestiCult Organoid Growth Medium (Stemcell, 06010) was added to each well. Then the plates were transferred to humidified 37°C/5% CO2 incubators. Medium was changed every 4 days and organoids were passaged every 2–3 days. When passaging, cystic organoids were resuspended in 2 mL cold AdDF+++ and mechanically sheared through flamed glass Pasteur pipettes. Dense organoids were resuspended in 2 mL TrypLE Express (Invitrogen, 12,605,036) and incubated for 1–5 min at room temperature. Later, the organoids were mechanically sheared through flamed glass pasteur pipettes. Following the addition of 10 mL AdDF+++ and centrifugation at 300 rcf, dissociated organoids were resuspended in cold Matrigel and seeded as above at ratio1:2 to 1:3. Single cell suspension was initially seeded at a higher density and reseeded at a lower density after one week. Mycoplasma tests were done with the MycoAlert mycoplasma detection kit (Lonza, LT07-318) and all were negative.

Histology and Imaging

Tissues and organoids were fixed in 4% paraformaldehyde followed by dehydration, paraffin embedding, sectioning and standard HE staining. Images were acquired on a Leica Eclipse E600 microscope.

Drug Screening

Organoids were grown in 96-well plates and treated for 48 hours with nab-paclitaxel at concentrations of 0, 0.625, 1.25, 2.50, 5.00, 10.00 and 20.00 μmol/L, or with 5-FU at concentrations of 0, 12.50, 25.00, 50.00, 100.00, 500.00, 1000.0 μmol/L or with epirubicin at concentrations of 0, 3.125, 6.25, 12.50, 25.00, 50.00, 100.00 μmol/L. After 48 hours, the viability of organoid was measured by CCK8 Assay. Dose-response curves were calculated on the basis of the absorbance readings collected from the CCK8 assay relative to drug concentrations. Absorbance was normalized to the vehicle controls, and drug concentrations were converted to logarithms by using GraphPad Prism (GraphPad Software, San Diego, CA). The half-inhibitory concentration (IC50) was determined as the concentration at which a 50% loss of viability, relative to that of untreated cells, occurred. For each organoid line, the experiment was performed 3 times.

Immunofluorescent Staining

Organoids were seeded in eight-well chamber slides (Lab-Tek). On day 3 following plating, medium was removed and organoids were fixed with 4% paraformaldehyde. After washing, wells were blocked with wash buffer containing 10% horse serum. Then, mouse monoclonal anti-Ki67 (1:50, R&D), Mouse Monoclonal anti-LGR5 (1:100, Thermo Fisher) or rabbit polyclonal anti-TROY (1:100, Abcam) anti-bodies were incubated overnight. Alexa Fluor 555/488-tagged secondary anti-bodies (1:500, ThermoFisher) were then added. Slides were visualized and images were taken using Nikon Eclipse Ti-S.

Apoptosis Assay in Organoids

The organoids were digested to single cells, then staining with PI and Annexin V-FITC. Apoptosis rates of 3 organoids were evaluated using an Annexin V-FITC/PI staining kit. In brief, 3 organoids were seeded at a density of 2×10^5cells/well in 12-well plates and treated with 0.57 μM nab-paclitaxel for 24, 36 and 48 h. After treatment, the organoids were then dissociated to single cells and washed twice with cold PBS and harvested by centrifugation. The cells were resuspended in 100 μL binding buffer, stained with 5 μL Annexin V-FITC and 5 μL PI, and incubated in the dark for 15 min at room temperature. Before detection, binding buffer was added to the cells to a total volume of 500 μL. Finally, the apoptotic proportion of the treated cells was measured by flow cytometry analysis (BD Biosciences). For each experiment, 10,000 cells were recorded.

Statistics

Quanlititive variables were presented by mean ± SD. Data analyses were conducted using GraphPad Prism 6.

Results

Characteristics of the Patients

The whole research process is depicted in Figure 1A. Three patients were enrolled in this study, including 2 male and 1 female patients. All patients were diagnosed as gastric cancer through gastric endoscopy test. Patients received standard gastrectomy and post surgery pathology analysis confirmed all tumors as gastric adenocarcinoma, with moderate to poor differentiation. All patients were proven to be stage III tumors. Molecular pathology analysis revealed all samples as microsatellite instable (MSI) negative, HER2 low expression and EBV negative (Table 1).

Establishing and Passaging of Gastric Cancer Organoids

Gastric cancer tissues were collected immediately after the removal of the tumors in the operation room. Overall, three attempts had been made for the primary culture of gastric cancer organoid. Tumor tissues were thoroughly washed. Through the process of mechanical disruption, enzymatic digestion and straining over a 100 μm filter, suspension of viable cells was obtained. At last, the isolated cells were mixed with Matrigel and then the mixture was seeded onto the plate. The gel was allowed to solidify and the organoid culture medium was added.

For all three cultures, small cysts formed by viable cells were present on day 1 after seeding. On day 3 some spheroids could be observed, and on day 5 to 8 fully confluent organoids formed (Figure 1B). These organoids were passaged, expanded typically at a ratio of 1:2. Rho-associated coiled-coil containing protein kinase (ROCK) had been proved to promote apoptosis of tumor cells and addition of the specific ROCK inhibitor Y-27,632 indeed facilitated proliferation and long-term in vitro survival of tumor cells. In all, we established three normal gastric epithelial-derived and GC-derived organoid lines that readily expanded (Table 2). Time lapse movie showed that the normal gastric organoid formed a cystic structure with mucin like materials in the lumen, which frequently bursted and released the content (Supplement video 1). This video shows the growth of normal stomach organoid. Time lapse movie showed that the normal gastric organoid formed a cystic structure with mucin like materials in the

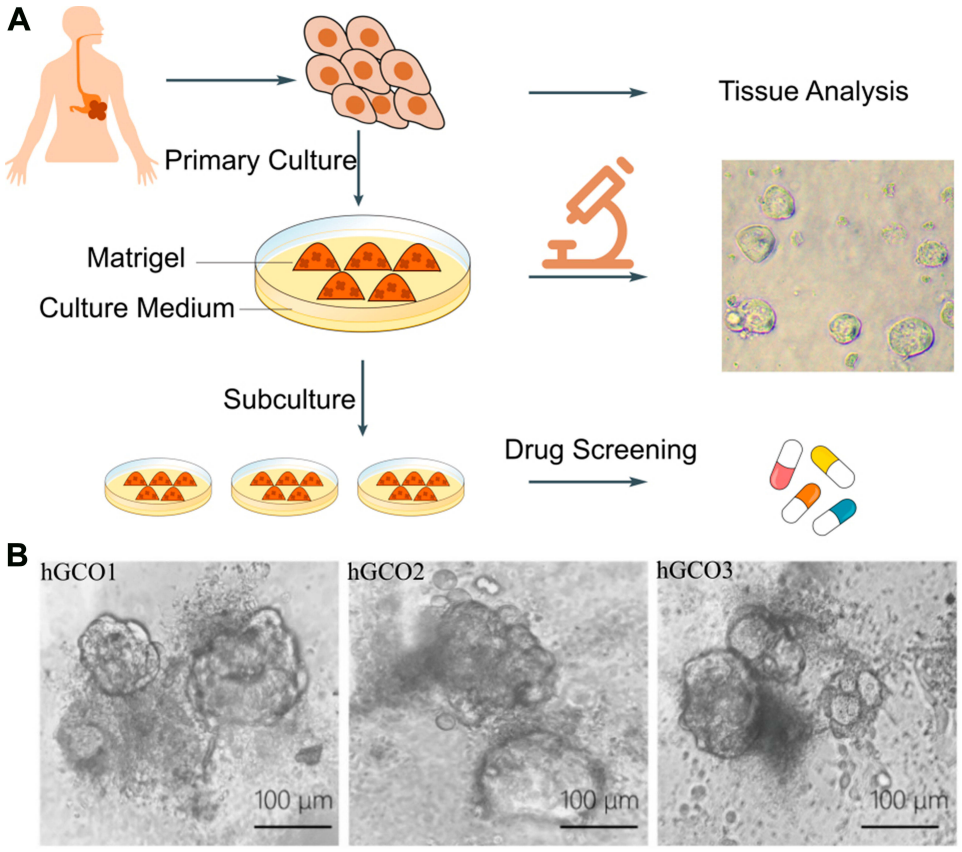

Figure 1 The morphology of established gastric cancer organoid under white field microscope. (**A**) Diagram depicting the procedure used to create organoids from surgical tissues. Surgical tissues obtained from patients were solid and therefore had to be sliced into small fragments before being digested into single cells, and seeded on the dish. Drug screening tests were performed after successful establishment of organoid culture. (**B**) Bright-field images depicting 3 lines of successful established gastric cancer organoids. Scale bar, 100 μm.

lumen. At some point, the cyst burst and released the content. Then the cyst sealed again.

Characterization of the Established Gastric Cancer Organoids

To test whether GC organoids match the histology of original GC tissue, we performed H&E staining of GC tissue and organoid sections. The phenotype of GC organoid agreed with the original GC (Figure 2A and B).

To validate the origin of organoid, we performed immunofluorescent staining to evaluate the expression of gastric markers. The gastric mucosal stem cell marker TROY,[18] the cell proliferation marker Ki67 and the gastrointestinal stem cell marker LGR5 were tested. We

Table 1 The Information of Patients

	Age/Gender	Histology	TNM Stage	MSI	HER2 IHC	EBV
1	54 M	Moderately and poorly differentiated adenocarcinoma	T3bN0M0	LOW	–	–
2	64 F	Moderately and poorly differentiated adenocarcinoma	T3bN0M0	LOW	–	–
3	73 M	Moderately and poorly differentiated adenocarcinoma	T4aN3bM0	LOW	–	–

Table 2 The Information of the Established Organoids

	Morphology	Passaging Interval (Days)	Passages	Successful Cryopreservation
1	Gastric adenocarcinoma	2	7	Yes
2	Gastric adenocarcinoma	3	6	Yes
3	Gastric adenocarcinoma	4	5	Yes

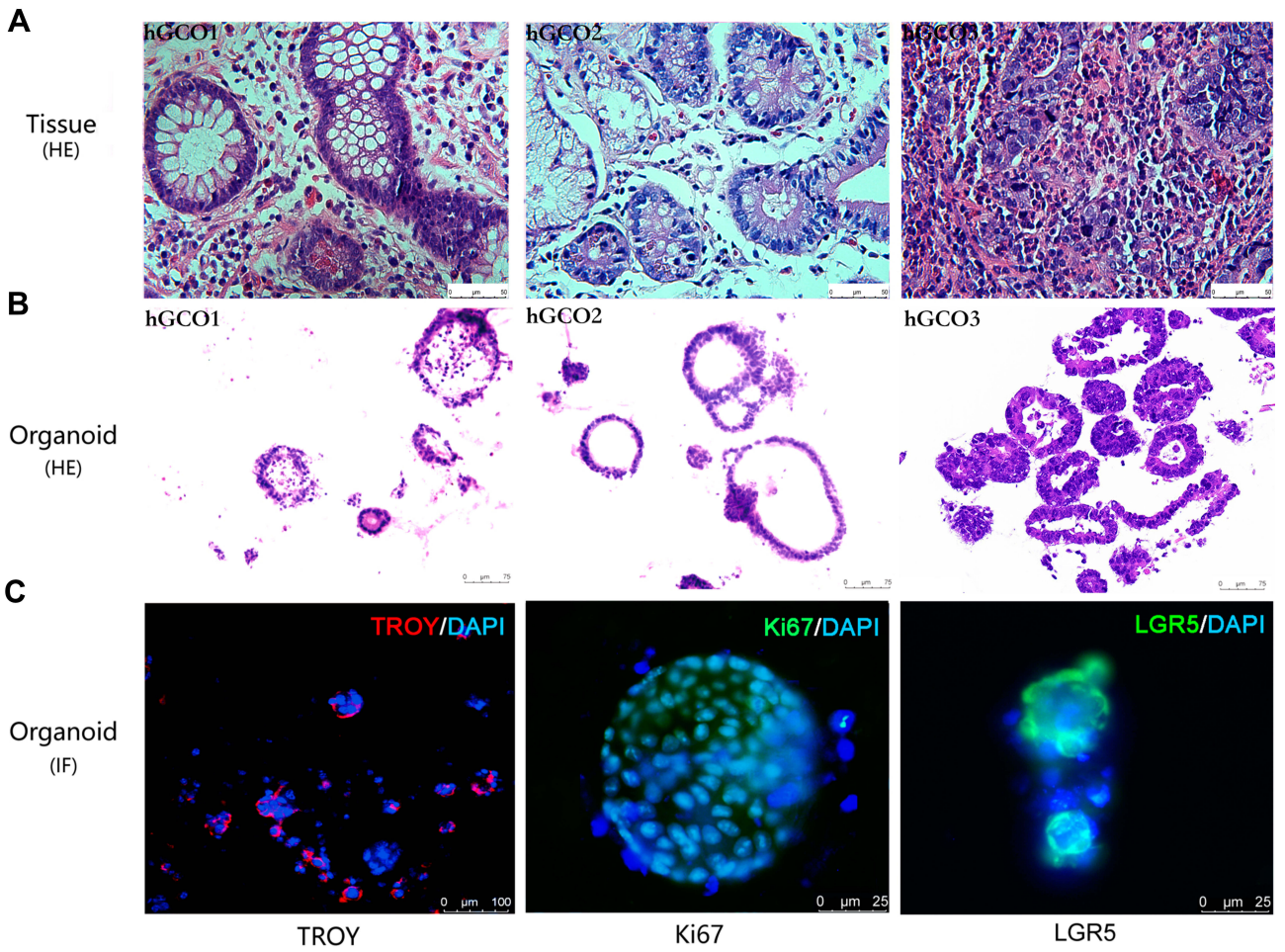

Figure 2 Representative image of gastric cancer tissues and organoids. Pathological analysis of the established gastric cancer organoids. (**A** and **B**) H&E staining of gastric cancer tissues (scale bar, 50 μm) and organoids (scale bar, 75 μm). Gastric cancer tissues and organoids show similar histological features. (**C**) Immunofluorescence staining of GC organoids. The left merged Figure shows the gastric stem cell marker TROY (red) and nuclear staining DAPI (blue) (scale bar, 100 μm), the middle merged figure shows the proliferation of gastric cancer organoids lines as measured by ki-67 (green) and nuclear staining DAPI (blue) (scale bar, 25 μm), and the right merged figure shows the stem cells in gastric cancer organoids lines as measured by LGR5(green) and nuclear staining DAPI (blue) (scale bar, 25 μm).

observed that Ki67, TROY and LGR5 were all widely expressed in gastric cancer organoids (Figure 2C).

Drug Sensitivity Testing: Nab-Paclitaxel Inhibits Human Gastric Cancer Organoids Proliferation

To explore the potential of using organoid as an avatar of the patients for personalized therapy, we tested drug sensitivity using the 3 cancer organoid lines. 5-FU and epirubicin are classic chemotherapeutic drugs widely used in gastric cancer.[19] Nab-paclitaxel, also known as nanoparticle albumin-bound paclitaxel, has been approved in many different tumor types. We tested each drug at six different concentrations (Figure 3). Our data showed that each organoid line exhibited a unique response to 5-FU, epirubicin and nab-paclitaxel (Table 2). The IC50 of 5-FU ranged from 28.32 to 91.59 μM. For epirubicin, the IC50 ranged from 7.6 to 25.85 μM in three organoid lines. Nab-paclitaxel showed lowerest IC50 values, ranging from 2.41 to 3.68 μM. In addition, we found nab-paclitaxel showed highest antiproliferative potency with a lowest effective dosage in all 3 organoid lines tested compared to 5-FU and epirubicin (Table 3). The difference in sensitivity to different drugs in three organoid lines may be attributed to the heterogeneity in each cancer organoid.

The Effect of Nab-Paclitaxel on the Apoptosis of Gastric Cancer-Derived Organoids

Annexin V- FITC/PI double staining and flowcytometry analysis demonstrated that after adding nab-paclitaxel to each organoid lines, the proportion of apoptotic cells in the

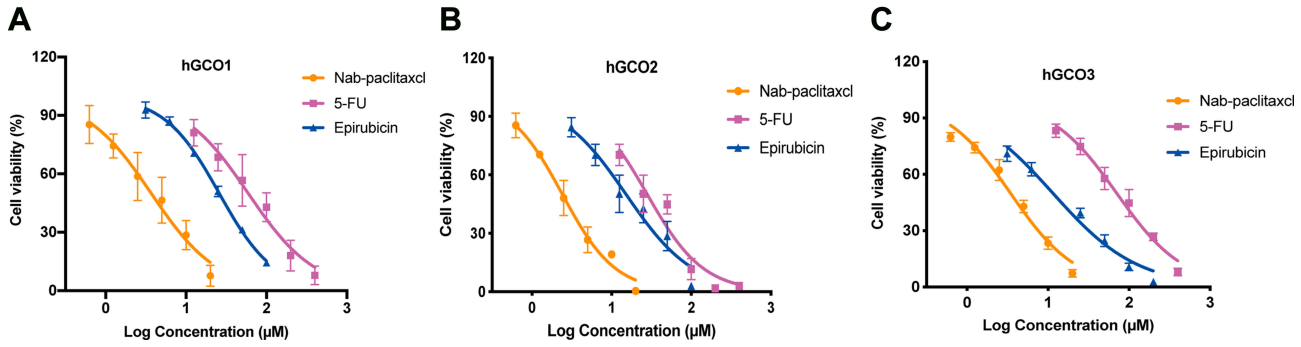

Figure 3 Nab-paclitaxel inhibits human gastric cancer organoids proliferation. (**A**–**C**) Three gastric cancer organoid lines (hGCO1, hGCO2, hGCO3) were used to evaluate drug sensitivity. Nab-paclitaxel, 5-fluorouracil and epirubicin were assessed in the organoids. IC50 values were lowest for nab-paclitaxel. Six different concentrations were used for each drug (x-axis), and cell viability of organoid was measured as a percentage of untreated control.

organoids significantly increased as time went by. For hGCO1, the overall percentage of apoptotic cells was 8.42% at 12 hours after treatment, which increased to 36.16% at 24 hours and 55.44% at 48 hours. For hGCO2, the percentage of apoptotic cells was 12.49% at 12 hours after treatment, which then increased to 27.20% at 24 hours and 57.65% at 48 hours. For hGCO3, the percentage of apoptotic cells was 9.70%, 25.21% and 58.99% after treatment for 12, 24 and 48 hours respectively (Figure 4A and B). In contrast, the non-treatment control hGCO1 only had 9.55% of apoptotic cells at 48 hours (Figure 4C).

Table 3 IC50 Values for huGCO Dose-Response Curves

5-FU			
log(inhibitor) vs normalized response	hGCO1	hGCO2	hGCO3
IC50 (μM)	72.99	28.32	91.59
95% Confidence Intervals			
LogIC50	1.81 to 1.92	1.40 to 1.51	1.87 to 2.06
IC50 (μM)	64.43 to 82.70	24.73 to 32.42	73.29 to 114.5
Epirubicin			
log(inhibitor) vs normalized response	hGCO1	hGCO2	hGCO3
LogIC50	1.41	1.18	0.88
IC50 (μM)	25.85	15.15	7.598
95% Confidence Intervals			
LogIC50	1.40 to 1.43	1.12 to 1.24	0.82 to 0.94
IC50 (μM)	24.91 to 26.82	13.11 to 17.51	6.57 to 8.78
Nab-paclitaxel			
log(inhibitor) vs normalized response	hGCO1	hGCO2	hGCO3
LogIC50	0.57	0.38	0.46
IC50 (μM)	3.68	2.41	2.91
95% Confidence Intervals			
LogIC50	0.50 to 0.64	0.34 to 0.42	0.40 to 0.53
IC50 (μM)	3.14 to 4.32	2.20 to 2.64	2.51 to 3.36

Discussion

Organoid can be cultured efficiently using human tissue and has the ability to simulate human diseases accurately.[20] It has been successfully used in various human diseases studies.[21] In addition, organoid has also been shown to be a good model to determine the optimal drugs for the patients.[8,22–25]

Most gastric cancer (GC) patients have locally advanced or distant metastatic diseases when the diagnosis is made for the first time.[26] According to the latest guidelines, for patients with metastatic gastric cancer, palliative chemotherapy is the only treatment option. Most patients with locally advanced gastric cancer may be suitable to undergo curative surgery, after which adjuvant chemotherapy was given to make sure the remnant cancer cells are destroyed.[26] Individual differences regarding molecular alterations (both genetic and epigenetic) and alterations in tumor microenvironment are associated with varying sensitivities to chemotherapy, which makes selection of the optimal drugs quite important.[27–29] Tumor tissue-derived organoids have been suggested to be a great preclinical model in predicting patient response to chemotherapy.[28] The advantages of the organoid model include: it can be successfully cultured from a small amount of tumor tissues; the successfully cultured organoids are consistent with original tumors in terms of genetic and histopathological characteristics; tumor tissue-derived organoids can be reliably used to determine the sensitive drugs.[30–33]

Paclitaxel, 5-Fu, and epirubicin are the commonly used drugs in routine clinical practice. Their high toxicity and poor effects in some patients should be a concern when

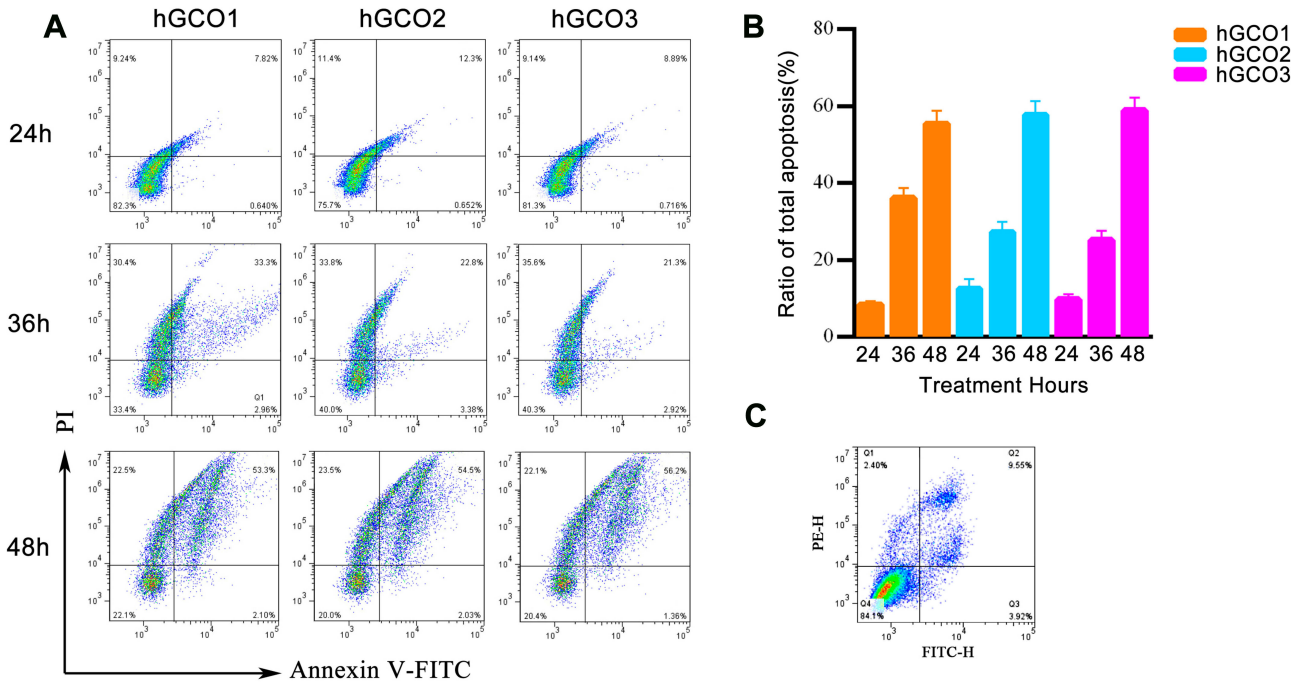

Figure 4 Apoptosis assays in 3 organoids treated with nab-paclitaxel by Annexin V-FITC/PI double staining. (**A**) Three organoids were treated with nab-paclitaxel for 24, 36, 48 hours, and the apoptosis rates were evaluated using flow cytometry analysis. (**B**) Quantitive analysis of apoptosis in 3 organoids treated with nab-paclitaxel. Each value represents the means ± SD of 3 separate experiments. (**C**) Flow cytometry analysis of the non-treatment control hGCO1 at 48 hours.

prescribing them to the cancer patients. The nanoparticle formulated nab-paclitaxel has been shown to be superior than docetaxel in treatment effect and toxicity profiles in breast cancer.[34] However, the role of nab-paclitaxel in gastric cancer remains ambiguous. So, in order to prove the feasibility of using nab-paclitaxel in gastric cancer, we performed this study. The results of this study demonstrated that nab-paclitaxel was more potent than other drugs in inhibiting gastric cancer growth, which reiterated the findings of our earlier study performed with gastric cancer cell lines.[35] The IC50s of the drugs measured by organoids are different from those determined with cell lines, which may partially attributed to the difference in tumor microenvironment between traditional 2D and organoid cultures. It also implies that, as a more faithful model than cell lines, organoid model is an ideal choice of drug sensitivity tests.

Several studies have been published that may explain why nab-paclitaxel is superiority over traditional drugs. Yardley Denise and colleagues have listed some potential mechanisms in a review article.[36] Firstly, an advantageous pharmacokinetic (PK) profile and the more efficient use of albumin-based transport result in 33% higher tumor uptake than traditional paclitaxel.[36] Secondly, the binding of albumin to secreted protein acidic and rich in cysteine (SPARC) may also contribute to the tumor accumulation of nab-paclitaxel.[36] Thirdly, the mechanistic synergy between nab-paclitaxel and other drugs may also play a role.[37,38] However, Kim et al demonstrated that the specific tumor uptake of nab-paclitaxel was not directly associated with SPARC expression.[39] Kinoshita et al argued that the improved anti-cancer effects of nab-paclitaxel were mediated by augmentation of EPR (Enhanced Permeability and Retention) effect and albumin-protein interactions using S-nitrosated human serum albumin dimer.[40] These theories should be further verified in GC patients since most of the aforementioned studies were performed with breast and pancreatic cancer.

A series of clinical studies on nab-paclitaxel in treating GC patients have been published over the past five years and most of these studies support the usage of nab-paclitaxel in treating GC.[15–17,36,41] In a Phase II study, Watson et al reported that nab-paclitaxel combined with FOLFOX brought promising results with manageable toxicity, which worth further investigation in Phase III studies.[15] Sato et al argued that the tri-weekly low dose of nab-paclitaxel therapy was effective in advanced gastric cancer patients with good tolerability and an acceptable margin of safety.[16] Bando H et al demonstrated that nab-paclitaxel plus ramucirumab combination therapy showed promising activity and manageable toxicities and

could be a useful second-line treatment option for advanced gastric cancer.[41] The findings of our in-vitro organoid study reiterated the results of those clinical trials. Large scale clinical trials may be warranted to gather more evidence.

In conclusion, our study demonstrates that nab-paclitaxel has stronger anti-tumor activity against gastric cancer compared to 5-FU and epirubicin in the organoid models we established. This evidence together with the results of multiple clinical trials suggest that nab-paclitaxel might be a safe and effective treatment option for gastric cancer. Besides, our study also suggests tumor tissue-derived organoid has the potential to be used as a versatile model for personalized drug screening and therapy.

Compliance with Ethical Standards

All experiments were in accordance with the ethics guidelines of the Human Ethics Committee of Seventh Affiliated Hospital of Sun Yat-sen University, and all patients provided written informed consent.

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Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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