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# CONSTRUCTING A CELL LINE-BASED TREATMENT MODEL FOR PANCREATIC ADENOCARCINOMA PATIENTS RECEIVING FIRST-LINE SECOND-LINE CHEMOTHERAPIES: A NOVEL METHOD IN PRECLINICAL STUDY

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**ABSTRACT** – ***Objective:*** Approximately 80% of patients with pancreatic adenocarcinoma (PA) are diagnosed with locally advanced, unresectable, or metastatic disease at presentation. First-, second-, and third-line chemotherapies are beneficial for patients. Gemcitabine (GEM)-based regimens played an indispensable role over the last two decades. There are several options for second-line treatment, such as a combination of oxaliplatin, fluorouracil (FU), and folinic acid (FOLFOX). However, the prognosis is still poor. Therefore, GEM as a targeted reference should be a reasonable potential approach. This study aimed to establish a first-line, second-line treatment model for future drug screening.

***Materials and Methods:*** We cultured SW1990 cells and compared the inhibitory effect on cell proliferation between the GEM-FOLFOX group, i.e., GEM treatment was followed by FOLFOX addition, and FOLFOX-GEM group, i.e., GEM was added after FOLFOX. Furthermore, we evaluated the inhibitory effect of both groups by changing onset of treatment with drugs, i.e., at early start (24 h after seeding cells) with that at delayed start (48 h after seeding cells), or by changing treatment duration i.e., long treatment duration (24 h) with that of shortened treatment duration (12 h). We quantified the expression of an antiapoptotic gene B cell CLL/lymphoma 2 (Bcl-2) using Real-Time quantitative polymerase chain reaction (PCR).

***Results:*** The GEM-FOLFOX group showed higher inhibition than the FOLFOX-GEM group, irrespective of delayed treatment or shortened treatment duration. Bcl-2 expression was more inhibited in the GEM-FOLFOX group.

***Conclusions:*** We developed a first-line, second-line treatment model for screening novel drugs that could have similar or better futuristic potential than GEM.

**KEYWORDS:** Gemcitabine, Pancreatic adenocarcinoma, First-line, Second-line.

## INTRODUCTION

PA is characterized by latent onset, rapid progression, and a high mortality rate, with a 5-year survival rate of 6-8%[1,2]. Only 10-20% of the patients diagnosed can be cured by surgical resections. Most patients benefit from first-, second-, or third-line chemotherapies and show a survival duration of 6-11 months[3-5]. Some of the first-line drugs are GEM and FOLFOX[6-8]. Patients with untreated advanced pancreatic carcinoma (APC)[7], when treated with FOLFOX-6 in a phase II trial, showed partial response (27.6%), stable disease status (34.5%), tumor growth control (62%), 4 months of median time to progression (TTP), and median survival of 7.5 months. GEM has been the primary first-line drug for ~20 years because it is better than FU[6]. Recent studies have shown that two combination regimens of GEM showed greater benefits for metastatic PA patients with good eastern cooperative oncology group (ECOG) performance status (PS) compared to GEM alone. A phase II/III clinical trial in 2011 compared a regimen of oxaliplatin, irinotecan, FU, and leucovorin (FOLFIRINOX) with GEM as first-line therapy in patients with metastatic PA[9,10] while a phase III study in 2013 compared combination of nanoparticle albumin-bound (nab)-paclitaxel and GEM (GEM/nab-paclitaxel) with GEM alone[4]. In the updated American Society of Clinical Oncology (ASCO) guideline of metastatic PA 2018[11], FOLFIRINOX and GEM/nab-paclitaxel were recommended for patients with ECOG PS of 0-1 as the first line, while GEM was recommended for patients with PS of 2. Meta-analysis of 12 randomized clinical trials showed a similarity in the number of PA patients between those with lower and higher ECOG PS (3006 *vs.* 2613)[12]. Therefore, ~50% of the total PA patients will benefit from GEM.

Second-line treatment commonly used FOLFOX[13-16], GEM, and Irinotecan. The efficacy and safety of second-line FOLFIRINOX treatment following GEM-based failure were studied and confirmed in a phase I/II trial[17]. In a phase II trial, the efficacy of nab-paclitaxel monotherapy was confirmed by patients with APC who progressed from GEM-based therapy[18]. ASCO guideline of metastatic PA[11] recommended GEM/nab-paclitaxel, FU/irinotecan, FU/oxaliplatin and FOLFOX for patients with ECOG PS of 0-1, and GEM or FU for PS of 2 in the second-line treatment. Another randomized phase II FIRGEM trial, second-line FOLFOX chemotherapy showed higher efficacy and acceptable toxicity in PA patients progressed from first-line GEM or sequential treatment[14]. A retrospective study evaluating the efficacy and safety outcomes of second-line FOLFOX-6 or FOLFIRINOX treatment for APC patients who experienced progression during first-line GEM-based treatment showed no significant differences in terms of progression-free survival (PFS) (26.29 weeks *vs.* 23.07 weeks) or overall survival (OS) from the start of second-line treatment (47.86 weeks *vs.* 42.00 weeks)[19]. Therefore, FOLFOX gained acceptance as a second-line treatment for APC and metastatic PA.

The aim of this study is to establish a first-line, second-line treatment model using SW1990 pancreatic cells which can be used for drug screening in the future.

## MATERIALS AND METHODS

### Materials

GEM hydrochloride (National Institutes for Food and Drug Control), Oxaliplatin (Shanghai Yongye Biological Company), Calcium Folinate (National Institutes for Food and Drug Control), FU (National Institutes for Food and Drug Control), SW1990 cell line (ATCC, Shanghai Zhewen Biotechnology Co., LTD, Fengxian, Shanghai, China), RPMI 1640 media (Gibco by Thermo Fisher Scientific, Suzhou, Jiangsu, China), fetal bovine serum (FBS) (Clark Bioscience, Claymont, DE, USA), 0.25%Trypsin-EDTA (Gibco by Life Technologies, Grand Island, NY, USA) for passage of cells, CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA) was used for detecting cell viability, TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract RNAs from SW1990 cells, RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) was used to transcribe RNAs into cDNAs, Real Time PCR Kit (Foregene, Chengdu, Sichuan, China) was used for amplification of target genes, primer (Sangon Biotech, Songjiang, Shanghai, China).

### Methods

#### *Cell culture*

SW1990 cells were cultured in RPMI-1640 media containing 10% FBS and kept in an incubator maintained at 37°C, humidified air, and 5% $CO_2$. The passage was done using 0.25% Trypsin-EDTA when the density reached 60-75%.

### *Cell proliferation assay (CPA), calculation of half inhibitory concentration (IC50)*

$5 \times 10^4$/mL (100 µL per well) cells were seeded in 96-well plates and kept in a 37°C incubator maintaining humidified air and 5% $CO_2$. After 24 h, drugs dissolved in phosphate buffered saline (PBS) with different final concentrations (FU 0.15625 µg/mL, 0.3125 µg/mL, 0.625 µg/mL, 1.25 µg/mL; GEM hydrochloride 1 µg/mL, 1.1 µg/mL, 1.25 µg/mL, 1.4 µg/mL; oxaliplatin 1.25 µg/mL, 2.5 µg/mL, 5 µg/mL, 7.5 µg/mL; calcium folinate 0 µg/mL, 2.5 µg/mL, 5 µg/mL, 10 µg/mL) were added to the culturing media (RPMI 1640 media containing 10% FBS) except the blank group (3 wells per drug) and then cultured further in the incubator. After 72 h 20 µL of CPA solution was added and incubated for 2 h. Then 25 µL of 10% sodium dodecyl sulfate (SDS) was added to stop the reaction. Optical density (OD) was measured using an enzyme-labeled instrument (detection wavelength: 492nm, reference wavelength: 630nm) and estimated inhibition ratio (100%) = $(1\text{-}OD_{experimental}/OD_{blank})\times100\%$. Figures were constructed (log-concentration *vs.* inhibition ratio) and IC50 was measured. Then we combined 3 drugs taking IC50 concentration of FU and oxaliplatin and a usually used concentration of calcium folinate and named it 1×FOLFOX (FU:0.407 µg/mL, calcium folinate: 2.5 µg/mL and oxaliplatin:1.309 µg/mL). Then CPA was tested by adding 0.4×FOLFOX, 0.5×FOLFOX, 0.6×FOLFOX, 0.8×FOLFOX, 1×FOLFOX, and the IC50 of FOLFOX was determined.

### *Study design: CPA in different sequential therapy timings*

Cells were cultured as above with methods 1-3 (Figure 1). Experiments were divided into 2 groups. In one group, cells were treated with GEM followed by FOLFOX (GEM-FOLFOX group), while cells of the other group were treated with FOLFOX followed by GEM (FOLFOX-GEM group). 0.396×FOLFOX was added to the FOLFOX-GEM group, 1.176 µg/mL GEM was added to the GEM-FOLFOX group, while no drugs were added to the blank group (3 wells per drug). Then the cells were kept in an incubator. After 24 h (or the corresponding duration as Figure 1), replaced with fresh media for all groups, 1.176 µg/mL GEM was added to the FOLFOX-GEM group, 0.396×FOLFOX was added to the GEM-FOLFOX group. Cells were incubated further. Then OD was detected, and the inhibition ratio was calculated. Cell proliferation inhibition effect between the two groups were compared.

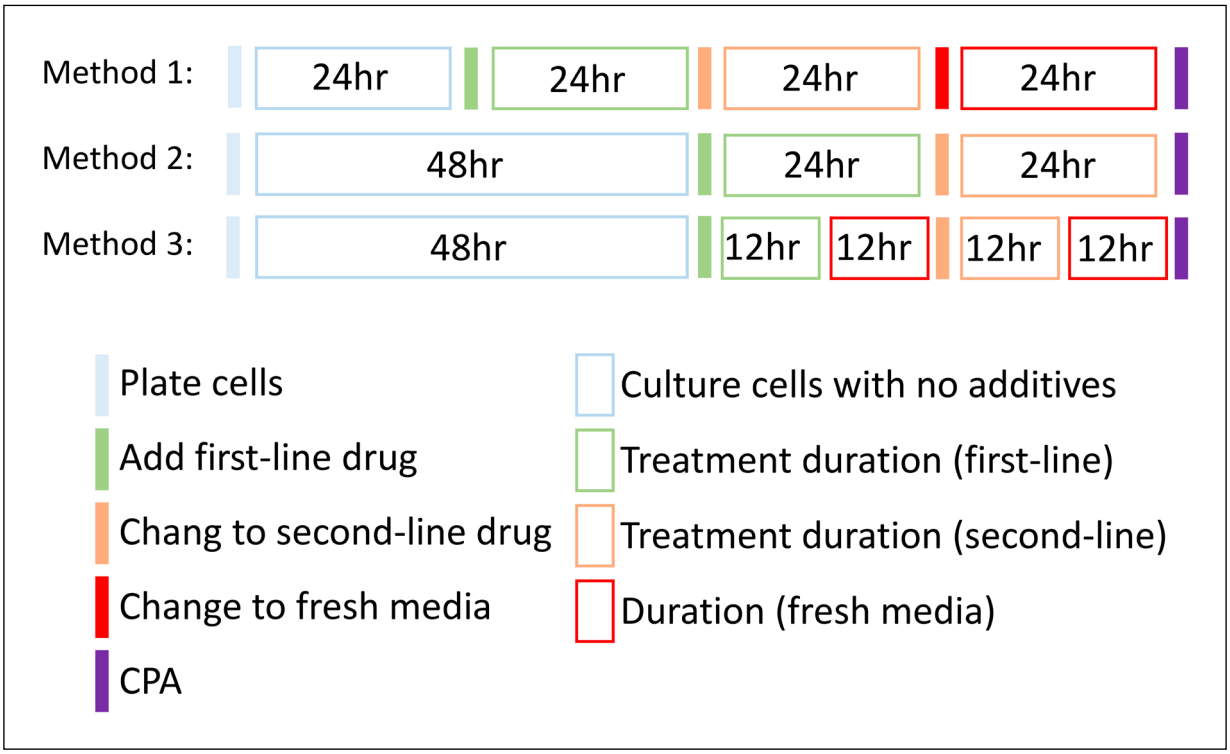



**Figure 1.** Schematic showing the time schedule of cell culture.

### *Real-Time quantitative PCR*

Total RNA was extracted from cells of each group using the TRIzol kit, and then the remaining genomic DNA was removed by DNase I. The RNA quality was checked by Nanodrop spectrophotometry. Reverse transcription was performed as mentioned in the datasheet. Real-Time PCR was performed by Applied Biosystems StepOne Real-Time PCR system (SN: 272005397) with the following conditions: Quantitation-Comparative $C_T$ $(2^{-\Delta\Delta Ct})^{20}$, 95°C for 3 min, followed by 40 cycles each at 95°C for 10 sec, 55°C for 10 sec and 72°C for 20 sec. Signals were collected at the end of each cycle. Forward primer of Bcl-2: 5'-TTG TGG CCT TCT TTG AGT TCG GTG-3'; reverse primer of Bcl-2: 5'-GGT GCC GGT TCA GGT ACT CAG TCA-3'. Forward primer of β-actin: 5'-TCC CTG GAG AAG AGC TAC G-3'; reverse primer of β-actin: 5'-GTA GTT TCG TGG ATG CCA CA-3'.

### *Statistical analysis*

Quantitative data are presented as mean ±standard deviation. Differences between the two groups were analyzed using the Independent Samples *t*-test of the Statistical Package for the Social Sciences (SPSS), version 22 (IBM Corp., Armonk, NY, USA). $p<0.05$ indicates statistical significance. Line graphs were performed using Microsoft Excel 2019 (Microsoft Corporation).

## RESULTS

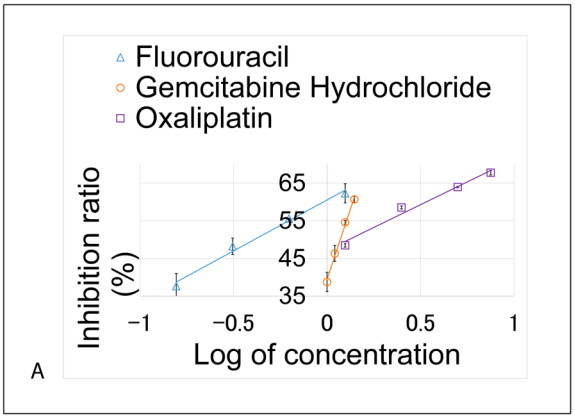
### Determination of IC50 of GEM, FU, oxaliplatin, and FOLFOX

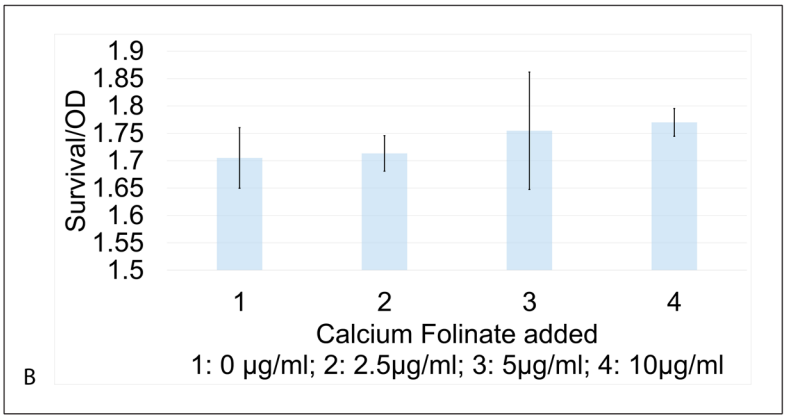
We determined the effect of four drugs on the proliferation of SW1990 cells using CPA. SW1990 cell line is derived from a metastatic spleen site of a PA patient. GEM, FU, and oxaliplatin showed concentration-dependent inhibitory effect (Figure 2 A). The inhibition effect of GEM reached 38.75%, 46.32%, 54.57%, and 60.67% at the final concentration of 1 μg/mL, 1.1 μg/mL, 1.25 μg/mL, and 1.4 μg/mL, respectively ($p = 0.017$, $p = 0.003$, $p<0.001$ between two adjacent groups, respectively). The IC50 of GEM was 1.176 μg/mL. The inhibition effect of FU reached 37.62%, 48.22%, 55.60%, and 62.32% at the final concentration of 0.15625 μg/mL, 0.3125 μg/mL, 0.625 μg/mL, and 1.25 μg/mL, respectively ($p = 0.01$, $p = 0.028$, $p = 0.044$ between two adjacent groups, respectively). The IC50 of FU was 0.407 μg/mL. The inhibition effect of oxaliplatin reached 48.47%, 58.55%, 63.98%, and 67.79% at the final concentration of 1.25 μg/mL, 2.5 μg/mL, 5 μg/mL, and 7.5 μg/mL, respectively ($p<0.001$, $p<0.001$, $p<0.001$ between two adjacent groups, respectively). The IC50 of oxaliplatin was 1.309 μg/mL. Calcium folinate is one of the commonly used drugs in chemotherapy regimens. We tested 2.5 μg/mL, 5 μg/mL, and 10 μg/mL of calcium folinate and found no inhibitory effect compared to the blank ($p = 0.833$, $p = 0.515$, $p = 0.138$, respectively) (Figure 2 B). We also tested the inhibitory effect of 0.4×FOLFOX, 0.5×FOLFOX, 0.6×FOLFOX, 0.8×FOLFOX, and 1×FOLFOX in a concentration-dependent manner and IC50 of FOLFOX was 0.396×FOLFOX ($p = 0.006$, $p = 0.005$, $p = 0.017$, $p = 0.011$ between two adjacent groups, respectively) (Figure 2 C). 0.396×FOLFOX comprised of FU (0.161 μg/mL), calcium folinate (0.99 μg/mL), and oxaliplatin (0.518 μg/mL).

### Cell proliferation inhibiting effect in the GEM-FOLFOX group

SW1990 cells were cultured according to methods 1-3 (Figure 1). We compared the inhibitory effect of the GEM-FOLFOX group with that of the FOLFOX-GEM group. In method 1, drug treatment was started 24 h after seeding cells (early start), while in method 2, it was started after 48 h (delayed start). GEM-FOLFOX group showed a higher inhibition effect than the FOLFOX-GEM group in both method 1 (71% > 63%, $p = 0.002$) and method 2 (32% > 28%, $p = 0.023$) (Figure 3 A). Method 1 of GEM-FOLFOX group showed a stronger inhibition effect than its method 2 (71% > 32%, $p<0.001$) (Figure 3 A). FOLFOX-GEM group also showed similar patterns (63% > 28%, $p<0.001$) (Figure 3 A). Our results indicated a stronger inhibition effect of GEM-FOLFOX group than FOLFOX-GEM group which correlated with an earlier start of treatment.

We also evaluated the effect of treatment duration on the inhibition effect in another experiment. We started treating cells at 48 h (method 1), 24 h (method 2), and 12 h (method 3) after plating cells



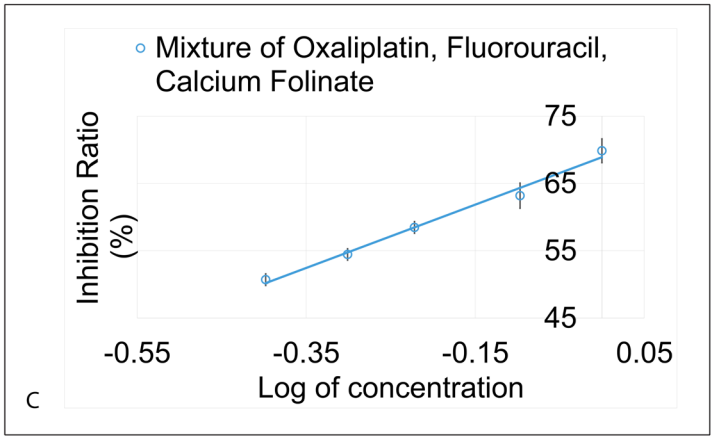




**Figure 2**. Cell proliferation assay (CPA) and determination of IC50s. (A) CPA of fluorouracil, gemcitabine hydrochloride and oxaliplatin. Final concentrations (fluorouracil: 0.15625 μg/mL, 0.3125 μg/mL, 0.625 μg/mL, 1.25 μg/mL; gemcitabine hydrochloride: 1 μg/mL, 1.1 μg/mL, 1.25 μg/mL, 1.4 μg/mL; oxaliplatin: 1.25 μg/mL, 2.5 μg/mL, 5 μg/mL, 7.5 μg/mL) were tested. (B) Calcium folinate was tested at 0 μg/mL, 2.5 μg/mL, 5 μg/mL and 10 μg/mL. (C) CPA of FOLFOX (mixture of oxaliplatin, fluorouracil and calcium folinate). 1×FOLFOX (fluorouracil: 0.407 μg/mL, calcium folinate: 2.5 μg/mL, oxaliplatin:1.309 μg/mL). CPA was tested by adding 0.4×FOLFOX, 0.5×FOLFOX, 0.6×FOLFOX, 0.8×FOLFOX, and 1×FOLFOX.

(Figure 1). GEM-FOLFOX group showed a higher inhibition effect than the FOLFOX-GEM group both in method 2 (29% > 21%, $p = 0.011$) and in method 3 (25% > 8%, $p<0.001$) (Figure 3 B). Within GEM-FOLFOX group, method 3 showed a lower inhibition effect than method 2 (25% < 29%, $p = 0.146$) (Figure 3 B). A similar pattern was observed in FOLFOX-GEM group (8% < 21%, $p<0.001$) (Figure 3 B). These results indicated a stronger inhibition effect of GEM-FOLFOX group than FOLFOX-GEM group which correlated with longer treatment duration.

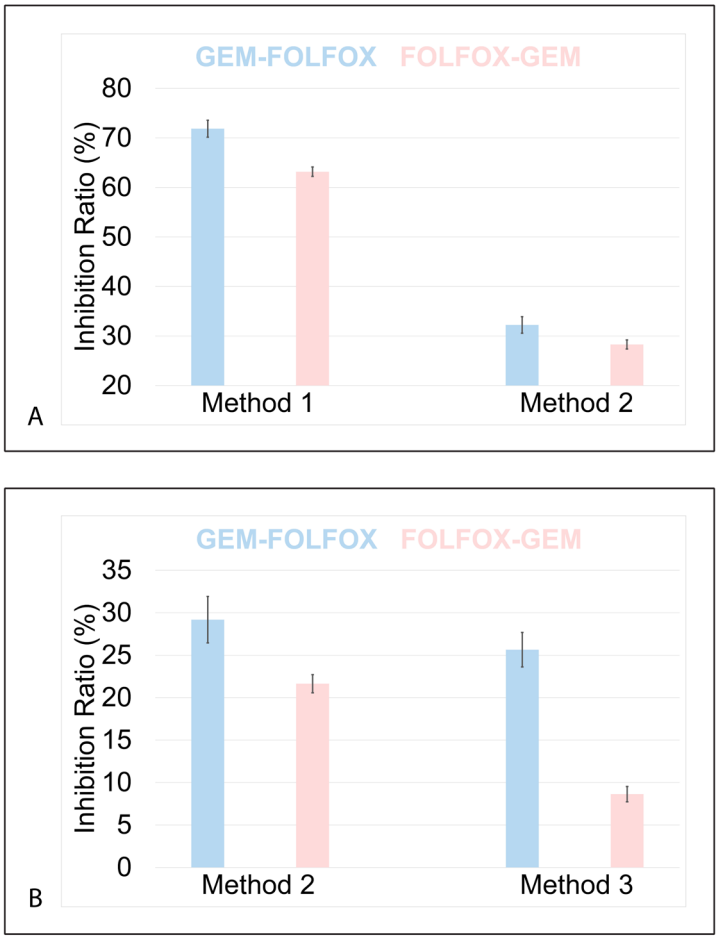



**Figure 3.** The inhibitory effect of cell proliferation for GEM-FOLFOX group and FOLFOX-GEM group with the culture method 1–3. (A) Two groups of cells were cultured by method 1 and 2. (B) The inhibitory effect by method 2 and 3.

These observations indicated that the GEM-FOLFOX group showed a steady stronger inhibition effect than the FOLFOX-GEM group irrespective of the start time of treatment (Figure 3 A) or treatment duration (Figure 3 B). GEM-FOLFOX group showed a weaker inhibitory effect due to delayed start of treatment.

## Expression of Bcl-2 gene in the GEM-FOLFOX group

To further investigate the mechanism behind the stronger inhibition effect of the GEM-FOLFOX group than the FOLFOX-GEM group, we performed Real-Time quantitative PCR using β-actin as an endogenous control. By using culture method 1, the expression of Bcl-2, an antiapoptotic gene in PA (including cell line SW1990)[21-24], was inhibited to 25% in the GEM-FOLFOX group compared to 58.7% in the FOLFOX-GEM group (Figure 4 A).

In an independent experiment, we tested how the start time of drug treatment affected the inhibitory effect. Within the GEM-FOLFOX group, culture method 2 with the start of treatment at 48 hours after plating (Figure 1) showed a weaker inhibition effect than the method which started at 24 hours after plating (Figure 4 A) (59.5% *vs.* 25%) but still stronger than FOLFOX-GEM group with the same start of treatment as culture method 2 (59.5% *vs.* 97%) (Figure 4 B).

These observations indicated a high correlation between stronger inhibited expression of the Bcl-2 gene and stronger inhibitory effect of the GEM-FOLFOX group in comparison with that of the FOLFOX-GEM group whenever we start treatment with drugs. A stronger inhibitory effect of the GEM-FOLFOX group could result from the highly inhibited expression of the Bcl-2 gene.

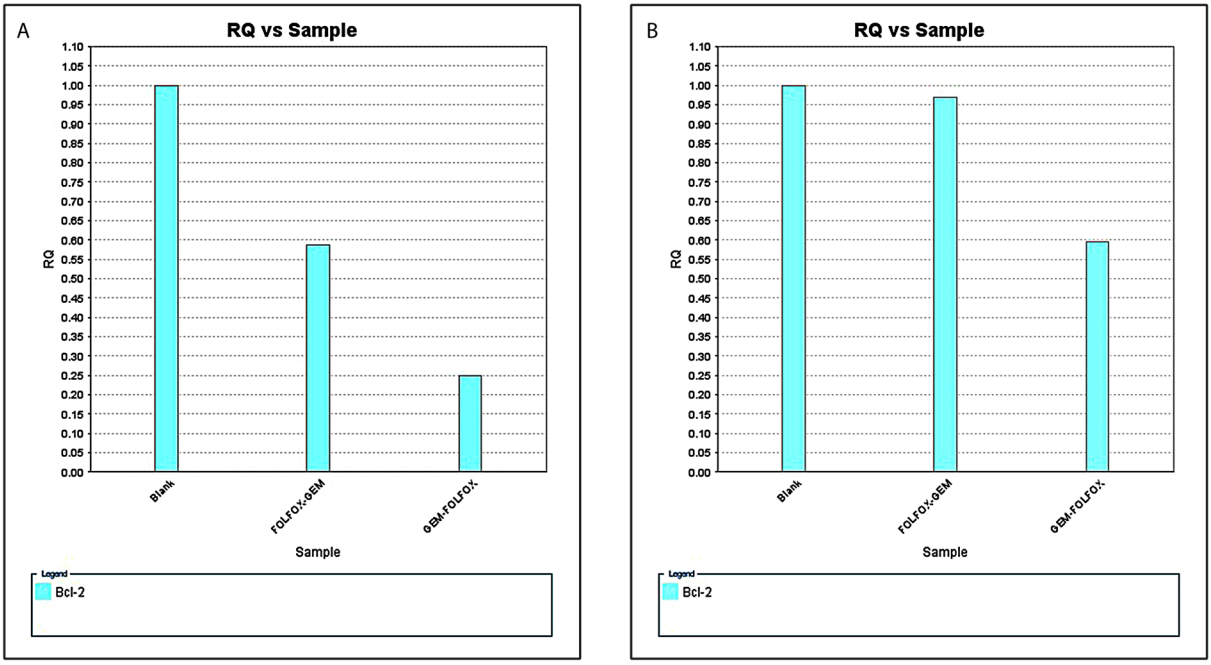



**Figure 4.** The inhibitory effect on Bcl-2 gene expression for GEM-FOLFOX group and FOLFOX-GEM group with the culture method 1–2 using Real Time quantitative PCR. (A) RNA samples were extracted from cells cultured by method 1. (B) RNA samples were extracted from cells cultured by method 2.

## DISCUSSION

To our knowledge, this is the first study to establish a first-line, second-line treatment model for human PA.

Most patients with PA usually receive second-line chemotherapy after the first-line. However, the effect of chemotherapy is not satisfactory. Currently, FOLFIRINOX and GEM/nab-paclitaxel are the main first-line treatments but are limited to metastatic cancer patients with good ECOG PS[25-27]. For patients with ECOG PS of 2, GEM is still the main solution[28]. As a second-line treatment, the most commonly used regimen involves cross-transitioning to GEM-based or FU-based treatments if FOLFIRINOX or GEM/nab-paclitaxel, respectively, was used before in the first-line. However, this regimen is restricted to patients only with good ECOG PS. For patients with ECOG PS of 2, FOLFOX and GEM are good candidates. Notably, the number of PA patients with lower ECOG PS was similar to that with higher ECOG PS (3006 *vs.* 2613)[12]. Therefore, approximately half of the total PA patients are predicted to benefit from GEM, while the other half are predicted to benefit from FOLFIRINOX or GEM/nab-paclitaxel.

A phase II trial tested the effect of the sequential treatment of FOLFOX-6 and GEM, followed by adapted maintenance for APC[8]. This FOLFOX-GEM trial showed partial response (22%), stable disease (22%), tumor growth control (44%), median TTP (4 months), and median OS (10 months). Grade 3/4 toxicities under FOLFOX were neutropenia (25%), thrombocytopenia (9%), anemia (9%), and diarrhea (6%), while under Gem were neutropenia (12.5%), thrombocytopenia (6%), anemia (6%) and hand-foot syndrome (9%). A meta-analysis examining OS in PA patients treated with second-line 5-FU and oxaliplatin-based therapy after failing first-line GEM-containing therapy showed that 5-FU and oxaliplatin-based therapies remain an acceptable and alternative second-line treatment option for patients with PA and adequate PS (e.g., ECOG 0-1)[29]. Furthermore, FOLFOX-6 and FOLFIRINOX showed no significant differences in PFS or OS of APC patients who experienced progression after first-line GEM-based treatment[19]. GEM-FOLFOX first-line second-line strategy has reached near standardization in treating PA patients with poor ECOG PS[30], which accounts for approximately half of total PA patients[12]. Therefore, we studied GEM and FOLFOX. An *ex vivo-in vivo* correlation study in a patient-derived subrenal capsule PA xenograft (SRCPCX) model was a promising tool in the early assessment of tumor sensitivity to GEM[31]. A first-line, second-line treatment model was established to understand the systematic effect *in vitro*, and to check whether the consistency is random or determined by some basic natural factors. GEM has been a standard first-line drug for PA for about 20 years and is still effective in about half of total PA patients[12,30]. Clinically GEM-FOLFOX systematic effect is better than FOLFOX-GEM[30]. Our results showed

a steady stronger inhibition effect of the GEM-FOLFOX group irrespective of delayed treatment start or shortened treatment duration. In our study, the expression of gene BCL-2, a factor promoting metastatic progression and therapy resistance of pancreatic cells[21-24] was highly inhibited in the GEM-FOLFOX group. The stronger inhibition effect of the GEM-FOLFOX group could be due to the stronger inhibition of Bcl-2 gene expression, which is highly associated with anti-apoptosis of PA[21-24]. Thus, this treatment model can be used to reflect the strength of the systematic effect, including first-line and second-line sequential treatments to some extent. However, tolerance, side effect, overall survival, and some other aspects can only be obtained from clinical trials. This treatment model can be used as a reference to screen novel candidates similar to or better than single reagent GEM.

## CONCLUSIONS

This study demonstrated that a first-line, second-line sequential treatment model constructed for screening novel drugs similar to or better than single reagent GEM, could provide consistency with clinical treatment course.

### Authors' Contributions:

Lining Wang conceived and designed the study. Yang Song, Xiaoyun Han and Ting Zhang performed the experiments. Yu Wang, Yu Liu, Yiping Mu and Yang Zhang collected and analyzed the data. Lining Wang, Yu Wang, Yu Liu, Yiping Mu and Yang Zhang drafted the manuscript. Lining Wang, Yu Wang, Yang Song, Xiaoyun Han and Ting Zhang contributed to the data interpretation and discussion. All authors read and approved the final manuscript.

### Availability of Data and Material:

The data and material are available by corresponding author upon request.

### Conflict of Interest:

The authors of this study declare that they have no competing interests.

### Ethics Approval and Informed Consent:

Not Applicable.

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