Ars Pharmaceutica
https://revistaseug.ugr.es/index.php/ars
E-ISSN: 2340-9894 ISSN: 0004-2927

doi: 10.30827/ars.v63i1.22390
Originales Breves

Citotoxicidad diferencial según el régimen de quimioterapia contra las células madre del cáncer de páncreas: estudio preliminar in vitro

Differential chemotherapeutic regimen cytotoxicity against pancreatic cancer stem cells: a preliminary in vitro study

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Recibido: 09.10.2021
Aceptado: 24.11.2021
Publicado: 20.12.2021

Financiación

This work was supported by funds from group CTS-107 (Andalusian Government). F. J. Q. acknowledges the FPU2019 grant from the Ministerio de Educacion Ciencia y Deporte y Competitividad (Spain).

Conflicto de intereses

No conflicts of interest to declare.

Agradecimientos

We are grateful to Scientific Instrumentation Center (CIC) from the Granada University.

Resumen

Introducción: El tratamiento del cáncer de páncreas en estadios avanzados se basa en diferentes regímenes de quimioterapia. Las células madre cancerosas son responsables de la quimiorresistencia tumoral y la recurrencia tras tratamientos en etapa adyuvante y metastásica. El objetivo de este artículo fue evaluar cómo estos regímenes quimioterapéuticos afectan a la proporción de células madre cancerosas y la expresión de sus marcadores.

Método: Utilizamos la línea celular de adenocarcinoma pancreático PANC-1 como modelo para aplicar diferentes protocolos quimioterapéuticos (monoterapia y terapia combinada) utilizando 5-Fluorouracilo, Oxaliplatino, Irinotecán, Gemcitabina y Abraxane.

Resultados: Tras analizar mediante RT-qPCR diferentes marcadores de células madre tumorales (SOX2, OCT4, CD133, CD44 y CD24) en células de cáncer de páncreas tratadas con diferentes protocolos quimioterapéuticos, el Oxaliplatino y la Gemcitabina en monoterapia fueron los quimioterápicos que seleccionaron en mayor medida las células madre cancerosas mientras que el protocolo FOLFIRI las disminuyó.

Conclusiones: En cuanto a la selección de marcadores, ha sido mucho mayor en el caso de Gemcitabina en monoterapia. En conclusión, estos hallazgos podrían mejorar y personalizar la terapia del cáncer de páncreas.

Palabras clave: cáncer de páncreas; céulas madre tumorales; quimiorresistencia; protocolos quimioterapéuticos; terapia personalizada.

Abstract

Introduction: Pancreatic cancer treatment in advanced stages is based on different chemotherapy regimens. Cancer stem cells are responsible for tumor chemoresistance and recurrence in adjuvant and metastatic settings. The objective of this article was to evaluate how these chemotherapeutic regimens affect the proportion of cancer stem cells and the expression of stemness markers.

Method: We used the pancreatic adenocarcinoma cell line PANC-1 as a model to apply different chemotherapeutic protocols (monotherapy and combined therapy) using 5-Fluorouracil, Oxaliplatin, Irinotecan, Gemcitabine and Abraxane.

Results: After analyzing different tumor stem cell markers (SOX2, OCT4, CD133, CD44 and CD24) in pancreatic cancer cells treated with different chemotherapeutic protocols by means of RT-qPCR, Oxaliplatin and Gemcitabine in monotherapy were the chemotherapies that selected the most cancer stem cells while the FOLFIRI protocol decreased them.

Conclusions: Regarding the selection of markers, it has been much higher in the case of Gemcitabine alone. In conclusion, these findings could improve and personalize pancreatic cancer therapy.

Keywords: Pancreatic cancer; cancer stem cells; chemoresistance; chemotherapeutic protocols; personalized therapy.

Highlights

Recurrence and chemoresistance in pancreatic cancer are largely related to cancer stem cells. The aim of this study was to determinate the effect of different chemotherapy protocols used in the clinic on tumor stem cell markers *in vitro*. The results show that gemcitabine monotherapy and oxaliplatin monotherapy increase these markers while the combination of 5-fluorouracil and irinotecan (FOLFIRI) decreases them. The expression of the different markers also changes depending on the treatment used.

Introduction

The current treatment of pancreatic cancer, the 9th most frequent and 4th deadliest type of cancer, remains ineffective. Although surgical resection can be applied when the tumor is well defined (20% of cases)[1], most patients with pancreatic cancer are treated with chemotherapy. Pancreatic cancer has a highly aggressive nature and, in most cases, it is diagnosed in advanced stages of the disease (III and IV). In stage IV, even with chemotherapeutic treatment, usually based on Gemcitabine-Abraxane (GEM-ABRAX) or 5-Fluorouracil (5-FU)-Irinotecan (CPT-11)- Oxaliplatin (OXA) also named FOLFIRINOX, the average survival is around 10 months. In localized resectable stages, the 5-year relapse rate is approximately 60% in patients treated with adjuvant chemotherapy regimens such as FOLFIRINOX and 80% in those exclusively treated with GMZ[2–5].

One of the main causes for the failure of chemotherapeutic treatments in pancreatic adenocarcinoma (PAD) is the development of drug resistance. Accordingly, a limited cytotoxic diffusion within the tumor has been described, seemingly due the specific nature of the extracellular matrix of PAD, which shows high levels of hyaluronic acid and fibronectin that originate a hypoxic environment[6]. In addition, the presence of cancer stem cells (CSCs) in these tumors is considered a key factor for drug resistance. In fact, CSCs have been demonstrated to show higher levels of different proteins involved in carcinogenesis compared to non-cancer stem cells. These proteins include poly-ADP ribose polymerase (PARP), which is implicated in DNA repair; P-glycoprotein, BCRP and MRP, which participate in drug detoxification; or NFkB and STAT3, which are involved in anti-apoptotic mechanisms[7]. Moreover, CSCs could be responsible for the poor prognosis of metastatic pancreatic cancer and high relapse rates despite adjuvant treatment. Pancreatic CSCs are positive for CD133, CD24, CD44 and EpCAM[8]. Besides, multiple factors related to cell differentiation and stemness (e.g., Nanog, SOX2, Oct4) have been proposed to explain the high recurrence and aggressiveness of pancreatic cancer cells[9].

In this context, the main objective of this article was to analyze the cytotoxicity and antitumor capacity of different chemotherapeutic regimens on pancreatic CSCs in vitro, as well as to determine which regimens have the highest cytotoxicity and antitumor effects on certain subpopulations of CSCs.

Methods

Cell culture

The pancreatic adenocarcinoma cell line PANC-1, obtained from Center of Scientific Instrumentation of Granada University, was grown in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and ATB (antibiotic, streptomycin + amphotericin B) at 1%. Cell line was maintained at 37°C in an atmosphere containing 5% CO2.

In vitro proliferation assays

PANC-1 Cells were seeded in 24-well plates (8000 cells/well) and incubated overnight. Then, the different treatments, 5-FU, OXA, CPT-11, GMZ (Sigma Aldrich-Merck, Darmastadt, Germany) and Abraxane (nab-paclitaxel) (Celgene, New Jersey, EEUU) were dissolved in DMEM and were administered without FBS and ATB. In the same way, there were control wells. In brief, after 72h, plates were fixed with 10% acid trichloroacetic (TCA) for 20 minutes and washed with distilled water for 3 times. Once dried, they have been stained with sulforhodamine B, which binds to the basic proteins of membrane of the cells

that remain glued to the wells of the plate, those that were live in the trial. They have been stained for 20 min, after which they have been washed with 1% acetic acid three times. They have been left dry. Once dry, tris-base has been added to the wells for 20 min and then they obtained 100 µL that have been added to a 96-well plate to read with a spectrophotometer and the Ascent software at 492nm. Absorbance values and inhibitory concentration 50 (IC50) values were calculated.

RT-qPCR assay

To determine modulation of CSCs markers expression, a Real-Time PCR was carried out. RNA (1 µg) (RNeasy Mini Kit, Qiagen, MD, USA) was reversed transcribed with M-MLV reverse transcriptase (Sigma, Italy). RNA was transcribed to cDNA by retrotranscription assay and a qPCR was carried out using the primers for pancreatic stem cells markers CD24, CD44, CD133, SOX2 and Oct4 (Sigma Aldrich-Merck, Darmastadt, Germany). In addition, GADPH was used as an endogenous control. The PCR cycling program was: 50°C (2min), 95°C (2 min), 40 cycles of denaturation at 95°C (30 s), annealing at 56°C (30 s), and extension at 72°C (40 s), followed by a melting curve analysis (range 56–95°C) with increments of 0.5°C/ 5 s to assess the primer specificity. The target transcripts were independently normalized to GADPH (housekeeping gene), and the RNA of the T0 cells was used as the calibration control. The results were expressed on a logarithmic scale as fold changes (FCs), with the 2^(–ΔΔCt) method.

Results and discussion

Pancreatic CSCs are defined by simultaneous positivity for, at least, three stem cell markers among which are CD24, a small cell surface mucin; CD44, a surface receptor for hyaluronic acid and fibronectin and CD133 or prominin, a tyrosine phosphorylase that is located in lipid rafts[8]. Previous studies showed great resistance of CSCs from different tumor types to chemotherapeutic agents in monotherapy such as GMZ, Paclitaxel, 5-FU, CPT-11 and OXA. In fact, GMZ selected pancreatic CSCs[10], colorectal CSCs were resistant to OXA and CPT-11[11,12], and gastric and ovarian CSCs were resistant to 5-FU and Paclitaxel, respectively[13,14]. To our knowledge, this is the first study analyzing the modulation of stemness markers by RT-qPCR in pancreatic cancer cells after exposure to different drug concentrations (near to IC50) (Table 1) both for monotherapy (GMZ, ABRAX, 5-FU, OXA and CPT-11) and combined therapy (GMZ-ABRAX, FOLFOX and FOLFIRI).

Interestingly, we demonstrated a higher percentage of CSCs in monotherapy regimens that were especially higher with the use of OXA. In addition, GMZ in monotherapy significantly increased several stemness markers (Figure 1.B). These results suggested that GMZ in adjuvant monotherapy could favor the presence of CSCs and tumor recurrence. By contrast, FOLFOX and FOLFIRI regimes were associated with a lower percentage of CSCs (Figure 1.C). FOLFIRINOX was not tested because of its high cytotoxicity. The association of GMZ and ABRAX did not significantly affect the proportion of CSCs.

In addition, an analysis of the RT-qPCR results showed that GMZ primarily selected positive CSCs for CD133, CD24, SOX2 and Oct4; OXA for CD44 and CPT-11 for SOX2. In addition, 5-FU reduced CSC marker expression whereas ABRAX did not seemingly select any of them. Regarding the combined regimens, GMZ- ABRAX selected pancreatic positive CSCs for CD133, CD24 and SOX2; FOLFOX showed a high tendency to select CD133 positive cells while FOLFIRI significantly decreased positive CSCs for CD133 and CD24 (Figure 1.C). These results suggest that the chemotherapeutic regimens showed a predilection for different CSC subpopulations with different stemness markers. This differential sensitivity could be used to develop personalized treatments after histological studies or liquid biopsy. Indeed, CD44 CSCs have been reported to show resistance to GMZ treatment[8]. However, our results showed that only OXA selected CD44 positive CSCs while GMZ increased the presence of CD24, CD133, Oct4 and SOX2 positive CSCs. Furthermore, a significant progression of pancreatic cancer has been shown in patients using GMZ-ABRAX treatment, probably by selecting for CD133-positive CSCs[8]. In this case, our results supported this hypothesis since GMZ-ABRAX selected cells positive for CD133, CD44 and SOX2.

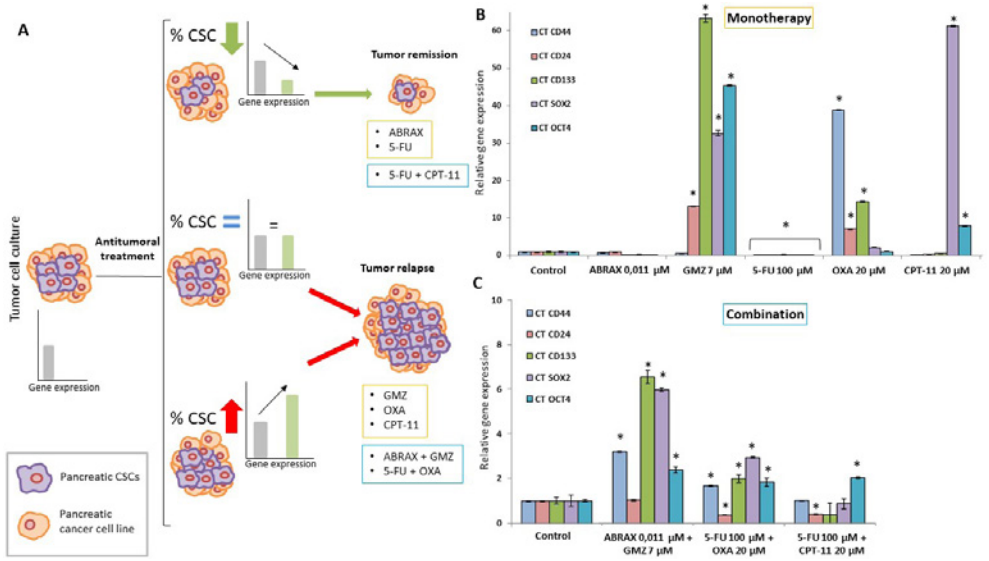

Figure 1. Modulation of CSCs markers in PANC-1 cells after exposure to monotherapy and combined therapy. A, Graphical representation of the determination of CSCs markers and their proportion in different treatments. B and C, qPCR analysis to determine the relative fold change of CD24, CD44, CD133, SOX2 and Oct4 mRNA expression in PANC-1 cells after exposure to monotherapy using 5-FU (100 µM), OXA (20 µM), CPT-11 (20 µM), GMZ (7 µM), and ABRAX (0.011 µM), and combined therapy using GMZ-ABRAX (20 + 0.011 µM), 5-FU-OXA (100 + 20 µM) and 5-FU-CPT11 (100 + 20 µM). Untreated samples (control) were established as calibrator. Data represents the mean value ± S.D. of triplicate experiences. * $p<0.05$.

Table 1. Ic50 value of different chemotherapies agents tested in PANC-1.

Chemotherapy	Ic50 (µM)
Abraxane	0.0096 ± 0.001
Gemcitabine	6.78 ± 0.42
5-Fluorouracile	97.27 ± 1.62
Oxaliplatin	18.32 ± 1.05
CPT-11	16.42 ± 0.98

Based on our *in vitro* results, the combination of the two monotherapies that did not select CSCs or cells with stemness markers (i.e. 5-FU and ABRAX) could show significant benefits in the treatment of pancreatic cancer. In addition, modulation of CSCs by the combined therapies tested in culture cells was observed. Accordingly, we could suggest conducting randomized controlled trials based on the combination of GMZ-ABRAX and FOLFIRI applied alternately (e.g. 2:1) in metastatic stages of pancreatic cancer. This would imply the use of treatment cycles focused on CSCs rather than merely the tumor mass, as is the case with GMZ-ABRAX. At tumor progression to GMZ-ABRAX, FOLFIRI could be a good chemotherapeutic regime because it reduces the CSCs population and stemness markers that are up-regulated by GMZ-ABRAX regime. With regard to adjuvant therapy, conducting clinical trials to demonstrate the superiority -or non-inferiority- of FOLFIRI over FOLFIRINOX would also be clarifying since pancreatic CSCs seem to be more sensitive to the first regimen. According, to this results the use of GMZ in monotherapy as adjuvant treatment in PAD could be counterproductive due to selection of several stemness markers and further studies should be developed to confirm these results.

In conclusion, pancreatic CSCs and different cell subpopulations with stemness have a differential sensitivity to chemotherapeutic regimens. Specifically, pancreatic CSCs showed the greatest sensitivity to

FOLFIRI compared to the rest of regimens analyzed in this study. These results could contribute to the development of personalized treatments and to delay tumor recurrence and progression in pancreatic cancer. In fact, this is the first *in vitro* study that tests the differential cytotoxicity of chemotherapeutic agents against different populations of pancreatic CSCs.

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