Gemcitabine Sensitivity on Podocalyxin Knockdown Pancreatic Cell Line SW1990

by

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Abstract

In this study, we tested cell survival following treatments of Gemcitabine (GE) in scrambled control and Podocalyxin (PODXL) knockdown pancreatic cancer cell line SW1990. After testing several major chemotherapeutic drugs with MTT cell survival assay, GE, brand name Gemzar, was shown to have a much higher cytotoxic effect on PODXL knockdown cells than control SW1990 pancreatic cancer cells. Upon observation, we suggested that knocking down PODXL could potentially reduce the resistance and/or increase the sensitivity of SW1990 to GE. With this hypothesis, we proceeded to test the expression of NF-κB targeted genes, which are indicator of cell survival mostly related to cell apoptosis, in both scrambled control and PODXL knockdown cells with and without GE treatments. The seven specific genes analyzed were cyclin D1, c-myc, bcl-2, bcl-xl, c-IAP1, cox-2 and MMP9. Apoptosis-related gene expressions were analyzed by reverse transcription-polymerase chain reaction (rt-PCR). Our results showed that five out of seven of the anti apoptotic genes were down regulated in PODXL knockdown and two out of seven of the anti apoptotic genes were significantly down regulated in PODXL knockdown following GE treatment. Combined data supports our hypothesis that PODXL knockdown cells are more sensitive to GE as a result of apoptosis induction.

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INTRODUCTION

Pancreatic cancer is one of the most deadly diseases in the world. Due to its resistance to most cytotoxic drugs, pancreatic cancer patients’ five-year relative survival rate is as low as 3 percent. [1] Such low percentage could be the result of suboptimal drug delivery, poor drug efficacy, insufficient cellular uptake, and chemotherapeutic resistance. In this study, a surprising increase in cell death was observed following treatment of GE in pancreatic cancer cell line SW1990 upon the knockdown of a transmembrane protein, PODXL.

PODXL is a transmembrane protein that is expressed in many types of human cells such as platelets and vascular endothelial cells. [2] First discovered in kidney, PODXL’s main function is the keep the unitary filtration barrier open. [3] It is upregulated in numerous types of cancers such as breast, colon, and pancreatic cancers. [4] PODXL has been identified as a functional E- and L-selectin ligand expressed by metastatic pancreatic cancer. [5] Data also shown that metastatic pancreatic cancer cells overexpress PODXL compared with nonmalignant pancreatic epithelial cells. [5] Yet, PODXL’s connection with GE resistance is still largely unexplored.

GE is a nucleoside analogue used as an anti-neoplastic drug. It causes apoptosis by replacing cytidine during DNA replication or by binding to ribonucleotide reductase (RNR). [6] Once transported through cellular
membrane, GE is phosphorylated into its active diphosphate and triphosphate form, which competes with the natural deoxycytidine-triphosphate analog for incorporation in DNA and RNA. This metabolism inhibits cytidine triphosphate synthase and terminates DNA synthesis. GE is currently the first-line agent for advanced pancreatic treatment. However, low protein binding of less than 10% as revealed by pharmacokinetic data low response rate of 20% indicate the need for improvement in overall GE performance. [7]

GE response in pancreatic cancer involves many intracellular pathways. In this study we specifically focused on the end terminus of the Phosphatidylinositol-3 kinase/AKT/NF-κB axis, the NF-κB targeted genes. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is anti-apoptotic transcription factor that promotes cancer growth. In short, GE exerts its cytotoxic effect in pancreatic cancer cells in vitro and in vivo by inhibiting NF-κB and its targeted genes cyclin D1, c-myc, bcl-2, bcl-xL, c-IAP1, cox-2 and MMP-9, which promotes cell death. [8,9,10]

In this work we focused on the expression of seven targeted genes of control and PODXL knockdown cells before and after treatment with 10 μM GE for 72 hours. Each of the seven genes listed influence the survival of pancreatic cancer cells, but the knowledge of the seven NF-κB targeted genes is not yet fully understood and their complete functions are still under investigation. Since this work mainly focuses on the NF-κB gene expressions, we provide a
comprehensive review of each gene with special regards to how they affect apoptosis.

NF-κB: cyclin D1, c-myc, bcl-2, bcl-xL, c-IAP1, cox-2 and MMP-9

*Cyclin D1* is a protein kinase that regulates cell cycle. It is overexpressed gene in breast carcinoma. [11] Overexpression of *cyclin D1* promotes growth in pancreatic tumor cancer cells and cyclin D1-overexpressing cells exhibit significantly reduced chemosensitivity to cisplatin. [12] This suggests that overexpression of *cyclin D1* could cause chemoresistance in pancreatic cancer due to its roles in promoting cell proliferation and inhibiting drug-induced apoptosis.

*C-myc* is another regulatory gene that regulates cell proliferation. In many cancers, mutated version of *c-myc* causes *c-myc* to be persistently expressed, leading to disrupted expression of many other downstream genes associated with cell proliferation. [13] *C-myc* is overexpressed in pancreatic cancers and renders them resistance to cisplatin. [14] Overexpressing *c-myc* could interfere with the process of apoptosis and leads to unregulated cell growth.

*Bcl-2* is an anti-apoptotic protein in pancreatic cancer and many other tumors. [15] *Bcl-2* is an integral membrane protein located on the outer
membrane of mitochondria that prevents apoptosis by blocking the release of cytochrome c, an initiation factor of apoptosis [16] In a multiple myeloma chemotherapy study, bcl-2 overexpression is associated to resistance to paclitaxel, but not GE. [17] Paclitaxel induces cell arrest at G2/M phase whereas GE induces cell arrest at S phase of the cell cycle. In this particular study, paclitaxel induced a down regulation of bcl-2 whereas treatment with GE did not affect bcl-2 expression. This suggests that bcl-2 might be irrelevant to GE resistance in pancreatic cancer. Although bcl-2 might not be directly influenced by GE treatment, its antiapoptotic character is noteworthy.

B-cell lymphoma-extra large (bcl-xL) is also a member of the bcl-2 family. Like bcl-2, bcl-xL is a transmembrane protein in the mitochondria, which is also a pro-survival protein that prevents the release of mitochondrial substances, such as many proteases, and eventually prevents apoptosis. bcl-xL protein level is a very useful tool to determine the apoptotic index. In a recent study analyzing the expression of Bcl-2, Bcl-xL, and Bax in pancreatic ductal carcinoma and their correlation to the extent of apoptosis, an upregulation of all the apoptotic regulatory molecules, especially bcl-xL, was observed. (The apoptotic index was determined by terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) essay) [18] Therefore bcl-xL expression could be used as an indicator of relative apoptotic rate in this GE study.
Baculoviral IAP repeat-containing protein 2 (c-IAP1), like bcl-2 and bcl-xL, is a prosurvival protein that inhibits apoptosis by interfering with the activation of caspases, which are a family of cysteine proteases that play essential role in apoptosis. In both neoplastic and non-neoplastic pancreatic cancer cells, overexpression of c-IAP1 is observed in the early stage of cancer progression. [19] In particular, c-IAP1 plays a role in the inhibition of TNF-alpha-induced apoptosis by inducing NF-κB. [19] Therefore its expression is expected to decrease in PODXL knockdown cells since they became more sensitive to GE.

Cyclooxygenase-2 (cox-2) is an enzyme responsible for inflammation and pain, which converts arachidonic acid to prostaglandin during inflammation. Prostaglandin E2, a prostaglandin derivative, can stimulate cancer progression. [20] It is upregulated in pancreatic ductal adenocarcinomas (PDAC). [21] However, the mechanism of how cox-2 promotes PDAC is unclear. Some has suggested that deregulation of the cox-2/PGE2 pathway appears to affect colorectal tumorigenesis by promoting tumor maintenance and progression, and encouraging metastatic spread. [22] In relation to apoptosis, inhibition of cox-2 in a hepatocellular carcinoma study induces apoptosis signaling, activation of caspases, and apoptosis originating from mitochondria. [23]

At last, matrix metalloproteinase 9 (MMP-9) is a protease that is involved in the breakdown of the extracellular matrix in normal physiological processes and plays a major role in pancreatic cancer growth and metastasis. [24] MMP-9 is
more associated with cancer cell invasiveness rather than apoptosis. However, 
*MMP-9* causes the recruitment of pericytes and pericytes invasion in primary 
tumor development during the degradation of extracellular matrix. [25]
Pericytes induced the antiapoptotic protein *Bcl-w* in tumor endothelial cells both 
in vivo and in vitro thereby protecting tumor cells from cytotoxic elements. [26]
Therefore, indirectly, *MMP-9* may regulate apoptosis either through the 
generation of extracellular matrix molecules or transactivation of cell surface 
receptors. [25] Data also suggests the anti-apoptotic roles of a numbers of *MMPs* 
in *vitro* including *MMP-7, MMP-9, MMP-10* and *MMP-15*. [27,28,29] These *MMPs* 
could also be related to apoptosis resistance in tumor cells by assisting or 
enhancing the early stages of tumor dissemination. [30,31] Therefore a decrease 
in *MMP-9* expression may be expected in PODXL knockdown cells against GE.
Table 1 summarizes the above information of each gene on apoptosis and the predicted expression upon GE treatment to the more sensitive PODXL knockdown pancreatic cancer cells.

<table>
<thead>
<tr>
<th>NF-κB Genes</th>
<th>cyclin D1</th>
<th>c-myc</th>
<th>bcl-2</th>
<th>bcl-xl</th>
<th>c-IAP1</th>
<th>cox-2</th>
<th>MMP-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptotic Effect</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>*</td>
</tr>
<tr>
<td>Expected Expression in GE-PODXL Condition</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

**TABLE 1: SUMMARY OF NF-ΚB TARGETED GENES EFFECTS ON APOPTOSIS. A NEGATIVE MARK INDICATES ANTI-APOPTOTIC AND THE ASTERISK INDICATES INDIRECT EFFECT. THE DOWNWARD ARROW INDICATES A DOWN REGULATION OF THE GENE EXPRESSION. GE-PODXL REFERS TO THE PODXL KNOCKDOWN CELLS TREATING WITH GE FOR 24 HOURS.**

**EXPERIMENTAL METHODS**

**CELL CULTURE:**

The pancreatic adenocarcinoma cell lines SW1990 was obtained from the American Type Culture Collection (Manassas, VA) and Pa03C was a generous gift from Dr. Anirban Maitra (Johns Hopkins School of Medicine, Baltimore, MD, USA). [5] SW1990 and Pa03c-PODXL knockdown cell line was provided by Dr. Matt Dallas. [5] SW1990 cells were cultured in DMEM supplemented with 10% FBS and 50 μg/ml gentamicin. Cells were harvested via mild trypsinization (0.25% trypsin/EDTA·4Na for 5 min at 37°C) and incubated at 37°C for 2 h to regenerate surface glycoproteins. [5]
**CELL SURVIVAL ASSAY:**

SW1990/Pa03c control and PODXL knockdown cells (5,000/well) were seeded in 96-well plates in duplicates and allowed to adhere overnight. After overnight incubation, the cells were treated with various concentrations of GE of 0, 0.1, 1, 10, and 100 μM for 72 hours. Cell survival was measured by a standard MTT assay.

**MTT ASSAY:**

Cell viability was evaluated using 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenyl tetrazolium bromide (MTT) test. After the treatment period as indicated above, the incubation media were removed and exchanged with 100 μl of fresh media containing MTT (0.2 mg/ml), and cells were incubated at 37°C for 3 hours. The media were removed, and formazan crystals were solubilized with 150 μl of DMSO. Absorbance at 570 nm was measured in triplicate using a microplate reader. [32]

**Reverse Transcription Polymerase Chain Reaction (rt-PCR):**

Control and PODXL knockdown cells and control and PODXL knockdown cells treated with GE for 24 hours were prepared in 6-well plates. Instead of 72 hours, we treated cells with GE for 24 hours to test gene expressions before
major cell death occurred. Upon 100% confluency, cells were washed with cold
PBS twice before extracting the mRNA with Trizol. Extracted mRNAs were
prepared at sample size of 5 μg in triplicate along with other necessary reagents
(RNase, primers, etc.). The reagents are iTaq™ Universal Supermixes from Bio-
Rad. Primers’ sequences were obtained from NCBI. Primers were obtained from
Invitrogen. GAPDH was used as a reference to all genes.

RESULT

SW-PODXL KNOCK DOWN CELLS ARE 13% MORE SENSITIVE TO GEMCITABINE THAN
CONTROL CELLS

To investigate the sensitivity of human pancreatic cancer cell line
SW1990 and its PODXL knockdown to anticancer drugs, we tested
chemotherapeutic agent GE for pancreatic cancer in a dosage dependent manner.
Cells were incubated for 72 hours to a range of each agent’s concentrations going
from 0.1 to 100 μM. Among all the different conditions, PODXL knockdown cells
exhibited increased sensitive to GE compared to the control cells. Shown in
Figure 1, differences in cytotoxic effect were observed in different
concentrations of GE treatment. There is 10 to 22 percent cell death differences
in different drug concentrations. Figure 2 is the supplementary data of another
pancreatic cell line Pa03c for the generalization purpose.
FIGURE 1: PANCREATIC TUMOR CELLS SW 1990 SENSITIVITY TO GE. CELL SURVIVAL ASSAY WAS PERFORMED USING MTT METHOD. CELLS WERE TREATED FOR 72 HOURS WITH INCREASING CONCENTRATION OF GE (0.1 – 100 UM) CELL VIABILITY WAS PRESENTED AS A PERCENTAGE OF UNTREATED CELL CONTROL VALUES. BLUE DOT MARK REPRESENTS SCRAMBLE CONTROL WHILE THE TRIANGLE MARK REPRESENTS THE PODOCALYXIN KNOCKDOWN CELL LINE.

FIGURE 2: PANCREATIC TUMOR CELLS PA03C SENSITIVITY TO GE. CONDITIONS SAME AS FIGURE 1.
**NF-κB GENE EXPRESSIONS**

We've shown that PODXL knock down is more sensitive to GE than control SW1990. In the following figures we present the NF-κB genes expressions in four conditions: SW control (SW SC), SW PODXL Knockdown (SW KD), SW control treated with GE (SW GE SC), and SW PODXL Knockdown treated with GE (SW GE KD). Tumor cells were treated with GE for 24 hours. NF-κB targeted genes are anti-apoptotic; therefore a more GE-sensitive cell line would expect lower NF-κB gene expressions. Our data agrees with the prediction except for a little deviation. The Results are shown as follows.

**FIGURE 3 CYCLIN D1 GENE EXPRESSIONS ON SW1990 PANCREATIC CANCER CELLS. FROM LEFT TO RIGHT: CONTROL, PODXL KNOCKDOWN, CONTROL WITH GE TREATMENT, AND PODXL KNOCKDOWN WITH GE TREATMENT.**
FIGURE 4 C-MYC GENE EXPRESSIONS ON SW1990 PANCREATIC CANCER CELLS. FROM LEFT TO RIGHT: CONTROL, PODXL KNOCKDOWN, CONTROL WITH GE TREATMENT, AND PODXL KNOCKDOWN WITH GE TREATMENT.

FIGURE 5 BCL-2 GENE EXPRESSIONS ON SW1990 PANCREATIC CANCER CELLS. FROM LEFT TO RIGHT: CONTROL, PODXL KNOCKDOWN, CONTROL WITH GE TREATMENT, AND PODXL KNOCKDOWN WITH GE TREATMENT.
FIGURE 6 BCL-XL GENE EXPRESSIONS ON SW1990 PANCREATIC CANCER CELLS. FROM LEFT TO RIGHT: CONTROL, PODXL KNOCKDOWN, CONTROL WITH GE TREATMENT, AND PODXL KNOCKDOWN WITH GE TREATMENT.

FIGURE 7 CIAP1 GENE EXPRESSIONS ON SW1990 PANCREATIC CANCER CELLS. FROM LEFT TO RIGHT: CONTROL, PODXL KNOCKDOWN, CONTROL WITH GE TREATMENT, AND PODXL KNOCKDOWN WITH GE TREATMENT.
FIGURE 8 COX2 GENE EXPRESSIONS ON SW1990 PANCREATIC CANCER CELLS. FROM LEFT TO RIGHT: CONTROL, PODXL KNOCKDOWN, CONTROL WITH GE TREATMENT, AND PODXL KNOCKDOWN WITH GE TREATMENT.

FIGURE 9 MMP9 GENE EXPRESSIONS ON SW1990 PANCREATIC CANCER CELLS. FROM LEFT TO RIGHT: CONTROL, PODXL KNOCKDOWN, CONTROL WITH GE TREATMENT, AND PODXL KNOCKDOWN WITH GE TREATMENT.
Cyclin D1 gene overexpresses in SW GE SC but had insignificant difference in expressions on other three conditions. C-Myc gene expresses the same in both control and gemcitabine treated cells, but greatly reduced in both PODXL knockdown conditions. Bcl2 gene expressions are very interesting because it decreased 1 fold in SW GE KD, which supports the argument that PODXL KD increases the sensitivity of SW cell line to GE. Bcl-xl gene expressions decrease for 2 folds in SW KD, but are also lowered in both GE conditions. Ciap1 gene expressions have no significant difference in GE treated and GE non-treated but show a consistent decrease in the KD conditions. Cox-2 expression is significantly lowered in SW GE KD, while still appears to have a consistent reduction in the KD condition compared to SC condition. MMP-9 gene expression decreases in both KD conditions though it has no direct apoptotic effect. Since all the NF-κB genes are pro survival to cell apoptosis, downregulated gene expressions in the SW GE KD condition would be favorable to our prediction.

**DISCUSSION**

The focus of this work is to discover the reason of increased sensitivity of PODXL knockdown pancreatic cancer cells to GE in mRNA level. PODXL is a transmembrane protein involved in hematopoietic cell homing, kidney morphogenesis, breast cancer progression, and epithelial cell polarization. [33] It is also a selectin binding ligand on pancreatic cancer cells. On the other hand, GE
is taken up by cells through the aid of human equilibrative nucleoside transporter 1 and 2 (hENT1, 2) and eventually incorporates into DNA to cause apoptosis through series of phosphorylation. We also know that GE resistance is connected to PI3K/Akt/ NF-κB pathways. There seems to be very little connection between PODXL and GE. However, in a very recent astrocytoma study, PODXL overexpression was shown to promote astrocytoma cell survival against chemotherapeutic drug temozolomide by significantly increased phosphorylation of Akt. [34] Interestingly, our work shows that down regulation of PODXL inhibits cell survival against gemcitabine. Instead of being a nucleoside analogue like gemcitabine, temozolomide damages the DNA by methylation and causes apoptosis. Though those are two separate studies with different cells and drugs, both indicate the significance of PODXL in cancer cell survival. Combining our result with the astrocytoma and temozolomide study, it is logical to suggest that PODXL expression influences both NF-κB and Akt in gemcitabine resistance. Further investigation is required to confirm and generalize this statement.

Another early study suggested that NF-κB activity grants resistance to pancreatic cancer cells while PI3K/Akt seem to be less important against gemcitabine. [35] In this study they alter the gemcitabine dosage from 0.4 to 20 μM treating five pancreatic cell lines and observed that NF-κB induction was dose-dependent while PI3K/Akt activity was dose-independent. In our NF-κB gene expression results, bcl-2, bcl-xL, and cox-2 showed reduction upon PODXL knockdown with GE treatment compare to control. We randomly tested gemcitabine concentration of 10 μM and the differences in gene expressions
among different conditions were significant. A GE dosage and time dependent assay on PODXL knockdown would be a plus to our work. For example, assessing NF-κB gene expressions by altering the GE concentration profile with fixed treatment time and vise versa. (Alter time, fixed concentration). Further work is required to confirm the correlation between GE concentration and chemoresistance due to the PI3K/Akt mechanism.

So far we have discussed what is happening inside the cell, now lets look at the uptake efficiency of GE and the possible correlation among hENTs and PODXL and GE. GE moves through cell membrane by the aid of hENT1 and hENT2. High expression of hENT1 in stage I to VI pancreatic patients showed improved overall survival upon treatment of GE. [36] However, a recent study reported that inhibition of hENT1 transporter does not affect the cytotoxicity of gemcitabine on resistant pancreatic cancer cells. Additionally, the common proposals for chemoresistance of most nucleoside analogs are the lack of intracellular transport proteins and the dysregulation of intracellular enzymes. [32] Therefore it is reasonable to suggest that PODXL expression affects GE resistance by interrupting the intracellular signaling pathways but a test for the cellular uptake of GE through the cell membrane could further confirm whether GE cellular uptake is affected upon PODXL knockdown.

In conclusion, we've shown downregulation of PODXL promotes cell death against gemcitabine and affects the mRNA expression of NF-κB targeted
genes. Future investigation on the Akt/PI3K signaling pathway would give a more complete picture of GE resistance in pancreatic cancer. In fighting chemoresistance, blocking key proteins could improve response to a common chemotherapy drug. In a paclitaxel study, it has been reported that stabilizing microtubules by blocking certain proteins could enhance the cytotoxic response. [37] We do not yet know the mechanism behind the increased cytotoxic effect on GE-PODXL, but the increased cytotoxic effect of GE-PODXL itself is already a very good start. Besides investigating the signaling pathways of this increased cytotoxic effect, an *in vitro* cytotoxicity test of GE on control SW1990 with pharmacological inhibition of PODXL would strengthen our work. Eventually this would lead to higher drug efficacy, improved IC50, and better overall survival rate to the gemcitabine chemo treatment in pancreatic cancer
REFERENCE


Bcl-2 expression in pancreas development and pancreatic cancer progression. The Journal Of Pathology, 194(4), 444--450.


CURRICULUM VITAE

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Education

Johns Hopkins University, Baltimore, MD
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Design & Research Experience

JHU Cell Engineering and Molecular Biology Lab, Baltimore, MD
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Graduate Researcher
  • Specializes in cell-based assay development for biomarker identification and mechanism of drug resistance in pancreatic cancer cells and gene targeting and profiling
  • Designed and led a team project of a group of 3 on investigation of drug sensitivity on Podocalyxin knockdown cell line
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Project in Design: Pharmacokinetics/ Pharmacodynamics, Baltimore, MD
Sep 2013 – May 2014
Project Leader
  • Developed a pharmacology model that predicts the relationship between smoking and drinking behaviors for heavy smoker/drinker
  • Collaborated with Biomedical Engineering department to improve current type II diabetes treatments in weekly progress review meetings and PowerPoint presentations

Biochemical Engineer Senior Product Design Project, Baltimore, MD
Sep 2012 - May 2013
Project Leader
  • Designed and transformed a snail slime-based product that targeted a niche market into an oral gel treatment for periodontal disease and preventative treatment with a larger addressable market
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Institute of Nanobiotechnology (INBT), Baltimore, MD  
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Johns Hopkins University Symphony Orchestra  
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- 10+ concert performances

Representative, Taiwanese Student Association  
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