Does Multidrug Resistance Protein 5 (MRP5) Contribute to Acquired Resistance to Gemcitabine in Human Pancreatic Cancer PANC-1 Cell Line?

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Attestation of Authorship

"I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgments), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning."

Signed:

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Date: 31.03.2023

Role of MRP5 in the Acquisition of Resistance to Gemcitabine in Human Pancreatic Cancer PANC-1 Cell Line

Abstract

Objectives: Pancreatic cancer is among the largest cause of cancer-related deaths in New Zealand with a grim prognosis of approximately 3-5 months if left untreated. The present research aimed to generate gemcitabine resistant PANC-1 cell lines, determine sensitivities, and investigate changes in the expression of a key ABC transporter (ABCC5).

Method: PANC-1 cell cultures were grown and split in preparation for gemcitabine treatment. Increasing concentrations of gemcitabine (20 nM, 100 nM, 200 nM, 2000 nM, 4000 nM) were added to respective vials of PANC-1 cells to determine growth and cell viability was calculated. Using GAPDH as an internal control, the relative gene expression of ABCC5 was analysed by using quantitative real-time PCR (qRT-PCR).

Results: PANC-1 cell viability increased as gemcitabine concentrations increased showing that PANC-1 cells gained resistance to treatment. Additionally, post-qRT-PCR analysis, a fold change of 1065 was determined for the expression of the ABCC5 gene suggesting a statistically significant increase in the upregulation of ABCC5 mRNA transcripts in PANC-1 cells treated with increased gemcitabine concentrations.

Conclusions: Accordingly, it seems that gemcitabine treatment induced its resistance in a commonly used pancreatic cell line by upregulating the expression of proteins that aid in the efflux of anti-cancer medication. These results shed some light on the unknowns of chemotherapy drug effectiveness and may explain a cause for common chemotherapy medication resistance.

Keywords: PANC-1, Pancreatic cancer, ABCC5, MRP5

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Additionally, I want to take a moment to remember and honor the memory of my dear mother. Though she is no longer with me, her memory continues to inspire and uplift me each day.

Introduction

Pancreatic cancer is the fourth leading cause of cancer-related mortality and prevalence rates have steadily increased since the early 2000s. In 2020, approximately 500,000 deaths were caused by pancreatic cancer worldwide accounting for 7% of all cancer-related deaths (Hu et al., 2021). According to the GLOBOCAN age-standardized cancer observatory, there were 683 incidences of pancreatic cancer in New Zealand in 2020 with 592 deaths making pancreatic cancer one of the highest leading causes of cancer-related deaths in New Zealand (Sung et al., 2021). According to Phillips et al. (2002), there are variations in age-standardized rates (ASR) among different ethnic groups. The study showed that Maori had higher rates (7.3/100,000) compared to Pacific (6.4/100,000) and Other (5.6/100,000) ethnic groups. With a notoriously poor long-term survival rate, the average survival duration in New Zealand is 7 months. Additionally, Māori have poorer survivability than other ethnic groups (Phillips et al., 2002).

Pancreatic cancer incidences and fatalities vary globally. In developing countries, pancreatic cancer is not a common ailment, cancer-related mortality rate is ranked 8th and 10th as the most common cause of male and female mortality respectively. This decrease can likely be attributed to the low life expectancies of these nations and the lack of adequate diagnosis. In subsequent sections of this thesis, it will be highlighted that age often correlates with pancreatic cancer diagnoses. Conversely, in developed countries, pancreatic malignancy is a significant type of cancer, making up 3% of all cancer cases and 6% of all deaths related to diseases. The treatment options for pancreatic cancer in New Zealand are determined based on the stage of cancer and can include a variety of approaches. These may involve surgery, radiation treatment, chemotherapy, palliative care, or a combination of these treatments (Cancer Society., 2022)

First-line chemotherapy is the standard treatment for individuals with locally advanced or metastatic pancreatic cancer who have not received prior treatment. There are two frequently used chemotherapy combinations for this purpose: 5-FU, leucovorin, irinotecan, and oxaliplatin, and gemcitabine plus nab-paclitaxel. These chemotherapy regimens are known for their efficacy in specifically targeting pancreatic cancer cells and are commonly employed in clinical settings (Von Hoff et al., 2013)

Like most cancers, pancreatic cancer is staged as I to IV Stages by The American Joint Committee on Cancer (AJCC) staging framework, which integrates the TNM (tumour, lymph node, and metastasis) characterization. Surgical intervention is recommended for stage I/II pancreatic cancer patients followed by adjuvant therapy or Neoadjuvant treatment. Stages III (locally progressed) and IV (metastatic) pancreatic cancers are generally categorized as unresectable. However, it has been reported the benefits of neo-adjuvant therapy for certain borderline resectable tumours and consequently rendered them for surgical resections (Fossaert et al, 2022). As pancreatic cancer is usually diagnosed at the later stages, chemotherapy and chemo-radiotherapy are recommended for patients with advanced pancreatic cancer or with metastatic pancreatic cancer. Gemcitabine-based chemotherapy is preferred for Stage IV patients with good performance while for those with poor general health supportive therapy is performed. Pancreatic cancer is well-documented for its relapse after tumour resection with a 40-60 % chance of relapse in the patients after the surgery, and 2-3% mortality rate. Pancreatic cancer is well-known for its resistance to different chemotherapy drugs, both acquired and inherent (known as multi-drug resistance or MDR). This resistance often leads to limited survival rates Even when patients receive standard and preferred chemotherapy, complete recovery to a normal quality of life can take up to 2-3 months. The median survival period for the patients receiving a standard therapy is around

17– 27 months and the survival beyond 5 years of time is roughly 20% (Hu et al, 2021). Since pancreatic cancer is an intricate disease and thus, a better understanding of the MDR in pancreatic cancer may help render multidisciplinary approaches to increase the therapeutic efficacy in patients with pancreatic cancer.

For patients encountering relapse of the disease following resection, a confirmatory biopsy is recommended. If not obtained, chemo-radiation may be used for those with locally advanced disease. For patients with metastatic disease the treatment choices are impacted by the time period from the culmination of adjuvant therapy to the detection of metastases. In all instances of recurrent disease, a clinical trial is a preferred alternative, and the best supportive care is recommended. Since gemcitabine has been used as a front-line treatment for pancreatic cancer, most of the studies focusing on gemcitabine chemoresistance is for advanced pancreatic malignancy. The cause behind the susceptibility of pancreatic cancer to MDR (including resistance to gemcitabine) remains unclear. Nevertheless, clinical oncologists have observed that most of the patients treated with gemcitabine-based chemotherapy develop resistance to the drug. Such a clinical phenomenon implies that gemcitabine chemoresistance is a key obstacle to the effective management of pancreatic cancer. The downregulation of human equilibrative nucleoside transporter 1 (hENT1) has been initially attributed to the loss of gemcitabine sensitivity, followed by the changes in the expression of enzymes like deoxycytidine kinase (dCK) or cytidine deaminase (CDA) as scrutiny for gemcitabine resistance.

Gemcitabine Mechanism of Action

To better understand the gemcitabine resistance in pancreatic cancer it is essential to understand its mechanism of action. Gemcitabine (2, 2-difluorodeoxycytidine, dFdC) is an

analogue of deoxycytidine that has been tested as an experimental reagent against human leukaemia non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), ovarian and pancreatic cancer by using xenografted mouse models. In contrast to its structurally similar drug 1-β-D-arabinofuranosylcytosine (Ara-C), which cures leukaemia, gemcitabine has proven therapeutic efficacy against various types of solid tumours such as: NSCLC, ovarian and pancreatic cancer in subsequent clinical trials (Mini et al, 2006).

Gemcitabine has been indicated as the first-line treatment for pancreatic cancer, however, resistance to gemcitabine has been developed rapidly in all patients taking the drug. Gemcitabine resistance is both acquired and de novo, multifactorial in nature. The resistance mechanisms of gemcitabine have been classified into two major categories, namely pharmacodynamic and pharmacokinetic mechanisms. The pharmacodynamic mechanism is affected by multiple cellular therapeutic targets, which can be manipulated by changes in target affinities and apoptosis induction mechanisms. Pharmacokinetic mechanisms involve gemcitabine absorption, distribution, metabolism, and elimination at systemic and cellular levels. Gemcitabine is a hydrophilic compound and thus is taken up into the cells by nucleoside transporters (ENT1 (SLC29A1) and ENT2 (SLC29A2) and three concentrative nucleoside transporters (CNTs) CNT1 (SLC28A1), CNT2 (SLC28A2), and CNT3 (SLC28A3)) to approach its targets. Once in the cells, gemcitabine is converted to its active form by phosphorylation by deoxycytidine kinase (dCK). The resulting metabolite 2',2'-difluoro-2'-deoxycytidine monophosphate (dFdCMP) is then converted into its active diphosphate and triphosphate metabolites (dFdCDP and dFdCTP). Gemcitabine has a Km value of 4.6 μM for dCK, which is higher than the Km value for deoxycytidine (1.51 μM). This renders it a suitable substrate for dCK. Gemcitabine has a higher affinity with dCK when compared to Ara-C (Km values of 3.6 and 8.8 μM, respectively) and more importantly, its Vmax/Km ratio is more

favorable which leads to more efficient activation within cancer cells. Gemcitabine is acting as a prodrug with its active metabolites (dFdCTP) being inserted into the DNA strand during DNA synthesis, resulting in inhibition of synthesis and subsequently cytotoxic effect. Additionally, the intermediate metabolite of gemcitabine, dFdCDP, can prevent ribonucleotide reductase (RR) from functioning properly. RR is the key enzyme which produce dNTP (deoxyribonucleotide triphosphate, the DNA synthesis building blocks) for downstream DNA synthesis. Inhibition of RR by dFdCDP is also known as a 'self-potentiation' mechanism since the precursor of the active metabolite (dFdCTP) exerts a synergistic cytotoxic effect within cells. Thymidine kinase 2 (TK2), a mitochondrial enzyme has been associated with gemcitabine sensitivity (Mini et al, 2006) as it also phosphorylates gemcitabine but with lowered specificity and affinity compared to phosphorylating dCyD.

Since gemcitabine has multiple activities within cells and cellular kinetics profiles, multifactorial mechanisms can contribute to its loss of sensitivities, including the diminished uptake nucleoside transporters, inactivation and/or low activity of the dCK enzyme, efflux of gemcitabine active metabolites out of the cells by overproduced ABC transporters, and systemic inactivation of gemcitabine by cytidine deaminase (CDA). CDA is an enzyme responsible for the deactivation of gemcitabine by conversion into 2-deoxy-2, 2 difluorouridine (dFdU) with a Km value of 95.7 μM. While overexpression or superactive CDA could lower systemic exposure to gemcitabine, it was found that patients with deficient or very low CDA activity led to an increased systemic gemcitabine exposure and thus high toxicities in a prospective pharmacogenomic study (Ueno et al, 2007). Thus, targeting CDA may not be a valid clinical strategy for reversing gemcitabine resistance. Another inactivation pathway for Gemcitabine is also possible through the conversion (deamination) of dFdCMP to 2,2-diflurodeoxyuridine monophosphate (dFdUMP) followed by dCMP deaminase which

converts it to dFdU. This detoxification might limit the tumor accumulation of gemcitabine and/or its active metabolites and result in diminished overall survival of patients with pancreatic carcinoma.

High affinity/capacity nucleoside uptake transporters are well equipped in cancer cells to introduce hydrophilic nucleosides and gemcitabine into cells to exert their cytotoxic effects. Various transporters such as ENT1 (SLC29A1) and ENT2 (SLC29A2) and three concentrative nucleoside transporters (CNTs) CNT1 (SLC28A1), CNT2 (SLC28A2), and CNT3 (SLC28A3) are required for its entry into the cells. By using Oocytes microinjected with in vitro-transcribed RNAs of the above-mentioned nucleoside transporters, gemcitabine is taken up into Oocytes by the human concentrative nucleoside transporter 1 (hCNT1) and human equilibrate transporters 1 and 2 (hENT1 and hENT2) with Km values of 24, 160 and 740 µM, respectively; and there is very limited passive diffusion of gemcitabine across phospholipid bilayers (Mackey et al, 1999). More importantly, hCNT1 has been suggested to play a pivotal role in determining gemcitabine efficacy and treatment outcome since the greatest intrinsic activity was seen with the human concentrative nucleoside transporter 1 (hCNT1) (Vmax: Km of 0.24 pmol/µM/min) (Mackey et al, 1998). However, hENT1 mRNA and protein expression level has been a positive prognostic factor in pancreatic cancer patients receiving gemcitabine therapy. Although hRNT1 seems to be a promising clinical sensitivity predictor, further molecular signature genes and their roles in predicting the sensitivity and overall outcome of gemcitabine are warranted.

After passing through the cell membrane, gemcitabine is further transformed and phosphorylated into its two active forms - di (dFdC-DP) and triphosphate (dFdC-TP). The deoxycytidine kinase (dCK) enzyme is responsible for this. These are the activated derivatives

(dFdCTP) of gemcitabine which lead to the halting of the DNA polymerase activity by process of masked chain termination restriction. dCK-mediated conversion into its active forms is referred to as a "rate-limiting step," and lack in dCK expression could result in diminished activation of gemcitabine and thus a lower sensitivity to gemcitabine in pancreatic cancer cells. Clinically, dCK protein expression level was associated with overall survival rates which is consistent with its biochemical functions. In patients with higher dCK protein levels, the overall survival rate is significantly higher than those with lower dCK (Sebastiani et al, 2006). On the other hand, the downregulation of dCK by HuR (an RNA-binding protein) has been associated with a higher mortality rate among pancreatic cancer patients receiving gemcitabine treatment. In vitro, studies also support these clinical observations as overexpression of dCK significantly increased gemcitabine sensitivity.

Another potential prognostic biomarker for gemcitabine would be cytidine deaminase (CDA), which removes the NH2 group from the pyrimidine, facilitating the extrusion of uracil metabolite from the cell. This could cause a reduction in the active metabolite dFdCTP level. Some in vitro studies have suggested that inhibition of CDA significantly increased gemcitabine sensitivity by up to 54-fold in cancer cell lines. However, clinical genetic association studies noted that the effects of CDA genetic polymorphisms was associated with gemcitabine-induced hematological toxicity and neutropenia without apparent effect on anticancer efficacy (Ueno et al, 2007). Further studies may be needed to address the contribution of CDA to the clinical sensitivity of gemcitabine in patients with pancreatic cancer.

Since gemcitabine became available for pancreatic cancer chemotherapy treatment in 1997 (Burris et al., 1997), gemcitabine-based chemotherapy has been a standard and

preferred regimen for patients with metastatic pancreatic cancer or in the adjuvant setting (Klein et al., 2000) (Iacono, 2016). Combination chemotherapy with gemcitabine (e.g., + nab-paclitaxel) has been enhancing the efficacy of these drugs while reducing toxicity (Hu et al., 2016). Accumulating evidence suggests gemcitabine-based regimen has significantly improved overall survival and life quality in patients with metastatic pancreatic cancer.

Although gemcitabine has shown promise as a more superior chemotherapy treatment, there appears to be a lack of efficiency with this cytotoxic agent and a hasty development of acquired chemo-resistance to cancer cells occurring within a couple of weeks post-treatment initialization (Ioannou et al., 2016). Various factors may induce gemcitabine resistance including dysregulation of nucleoside transporters and nucleotide metabolizing enzymes, changes in the tumour microenvironment, and epithelial–mesenchymal transition (Zeng et al., 2019).

ATP-binding cassette (ABC) transporter-mediated drug resistance has been suggested to play pivotal roles in tumor resistance and result in therapeutic failure in cancer therapies. ABC transporters can pump therapeutic drugs out of cells against the drug concentration gradient by using the energy generated from ATP hydrolysis. The substrate of ABC transporters includes structure- and action-diverse drugs. This research focuses on the expression of an ABC transporter (drug efflux pump) gene, namely ABCC5, and whether they are upregulated with gemcitabine treatment. ABCC5 is a gene which codes for the multi drug resistance-associated protein 5 (MRP5). MRP5 is transporter protein that mediate the disposition of several physiological compounds and xenobiotics including nucleoside and its drug analogues (Jansen et al., 2015). An unpublished 2019 study revealed the possibility of silencing MRP5 using the CRISPR-Cas9 system may reverse gemcitabine resistance in

pancreatic cancer cell lines (Budge, 2019). These results suggest ABC transporters may be a novel target for modulation to countermand gemcitabine resistance in pancreatic cancer (Budge, 2019). However, little research exists on the role of ABCC5 in gemcitabine-induced resistance in pancreatic cancer. The main objective of this project is to assess ABCC5 mRNA transcript level in human pancreatic cancer PANC-1 cells treated with gemcitabine at sequentially increased concentrations.

Methods

PANC-1 Cell Line Culture

Pancreatic Cancer cell lines, Panc-1 were used in this project for in-vitro analysis. These cell lines were cultured using complete RPMI (Roswell Park Memorial Institute (RPMI) 1640 medium) medium supplemented with 10% (v/v) Foetal Bovine Serum (FBS), L-Glutamine (2 mM) (Life technologies, NZ), Penicillin (100 units/mL) and Streptomycin (100 µg/mL)) (Life Technologies, NZ) in a moistened atmosphere of 5% CO_2 at 37°C. All cell culture was carried out in aseptic conditions by using a biosafety hood (ESCO, Bio-Strategy®). Once the cell culture was 80-90% confluent, cells were split and sub-cultured.

Cell Viability

Once the desired cell confluency was achieved the cells were split by washing with pre-warm PBS briefly followed by trypsinization for 5 mins. The trypsinization was stopped by adding the equal volume of the medium (1:1) and spun for 5mins, 4°C at 500 $\times$ *g*. The supernatant media was discarded, and cells were re-suspended again in a complete medium. The cell viability was determined using the Trypan blue exclusion method. A 10µl cell suspension was mixed with 10µl of 0.4% Trypan blue (1:1). After this 10µl of the cell-dye mix was loaded on the Neubauer's chamber for counting; unstained (live) and stained (dead) cells

were counted. Cells were seeded in the new T75 flask with a cell density of 3-4 x 10^5 cells/T75 flask with 6-7 ml of complete RPMI medium. Cells were grown to achieve the confluency of ~80-90% (approximately 3 days). The confluent cell flask was again subjected to splitting, or the cells were used in different applications or experiments, e.g., drug cytotoxicity, Apoptosis, transfection, preparing more frozen cell stocks, etc. Cell culture over the passage number 20 was discarded.

Gemcitabine Treatment

Figure 1. *Flowchart of Method*

The stock solution of gemcitabine was purchased from Sigma at a concentration of 50 mM. A gemcitabine working solution of 400 µM was prepared by mixing 1 part (1µl) stock solution into 99 parts (99µl) of complete culture medium. The final gemcitabine concentration prepared were: 4000 nM, 2000 nM, 100 nM, 100 nM, and 20 nM (Figure 2). A blank medium was used as a control. The prepared solutions were pipetted into 6 cells and left for 96 hours for cell growth. After treatment, cells were observed under a microscope, and viable cell counts were made using a hemocytometer.

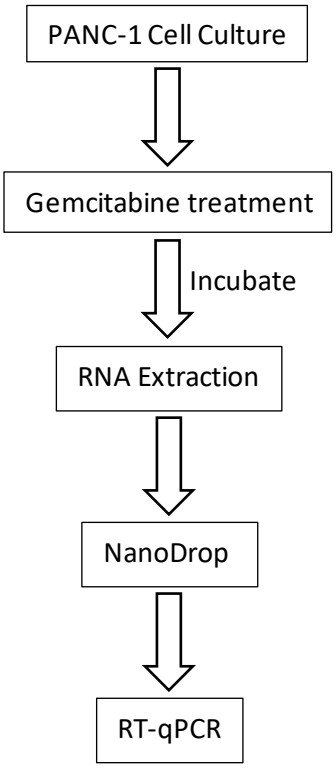


Figure 1: The flowchart illustrates the protocol method.

Generation of gemcitabine resistant PANC-1 cells

PANC-1 cells (~30-50% confluence) with complete culture media containing 20 nM of gemcitabine for 24 hours (Based on information gathered from (Avan et al, 2012)). Media changes were carried out every 2 to 3 days still containing this concentration of gemcitabine, and resistant clones were allowed to grow to 80% confluency before trypsinization. Cells are then reseeded at the original seeding density in media

containing twice the drug concentration (i.e., 40 nM). These escalations are then continued (40-> 80->160 nM) until a final concentration equal to 8-fold of the original IC50 concentration (i.e., 160 nM).

RNA Extraction and cDNA Synthesis

ABCC5 RNA was extracted from cell cultures using a commercial kit (Invitrogen PureLink RNA Mini Kit, Thermo Fisher Scientific) in accordance with the manufacturer's instructions. The quantity of extracted RNA was determined by a NanoDrop spectrophotometer at wavelengths 260 and 280 nm. Then, the total RNA (2 µg) was reverse transcribed into cDNA using a Reverse Transcription Kit according to the manufacturer's instructions.

Real-Time Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-qPCR)

Following reverse transcription, cDNA samples underwent RT-qPCR analysis by using the EvoScipt SYBER Green PCR Master I Mix and were performed on a LightCycler 480 system (Roche). The PCR conditions were: a 10-minute preincubation step (95°C), a 45-cycle amplification step (10 seconds at 95°C and 10 seconds at 53°C) followed by a 1-minute melting step (65°C). All samples were run in triplicate using GAPDH as a housekeeping gene. The relative quantification method or $2^{-\triangle\triangle CT}$ was used to analyse results due to its practicality, ease of use, and ability to represent data as fold change.

Results

Cell Viability

(Figure 2) *Cell Viability*

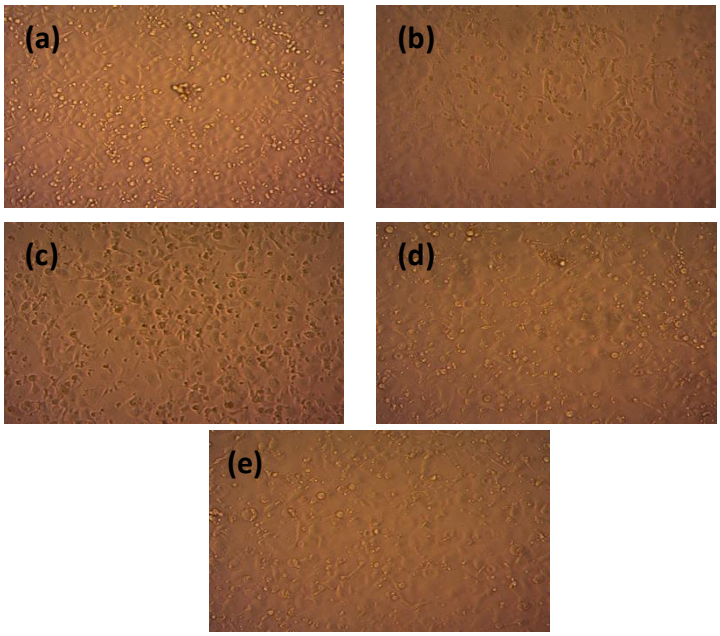


Figure 2: Cell Suspension Concentration: (a) 20 nM. (b) 100 nm, (c) 200 nM, (d) 2000 nM, (e) 4000 nM.

A trypan blue exclusion assay was conducted using a hemocytometer to determine the number of viable cells present in each of the varying gemcitabine concentration cell suspensions. Viable cells display a transparent or clear cytoplasm whereas apoptotic or nonviable cells display a darkened (blue) cytoplasm (Figure 2b). The 20 nM gemcitabine cell suspension showed a high cell viability with no observable apoptic cells suggesting that the concentration for cytotoxic effect had not been reached (Figure 2a). 100 nM gemcitabine treatment showed a lower cell viability with large numbers of apoptic cells (Figure 2b). A similar result was shown with 200 nM gemcitabine treatment (Figure 2c). No viable cells were observed. However, at 2000 nM and 4000 nM, cell viability increased (still less than that at 20 nM) (Figure 2d,2e). The number of apoptic cells decreased dramatically.

RNA Extraction Protocol

RNA extraction was carried out using the RNeasy® Mini Kit from Qiagen (Catalogue No. – 74106).

The PANC-1 cells cultured in a T25 flask (minimum cell no. 1 x 10^5) were used for RNA extraction (not more than 1 x 10^7 cells). The cells were washed with PBS and then detached by trypsinization. After centrifugation at 200 g for 5 minutes, the cells were collected as a

pellet in an RNase-free polystyrene tube. The cell number was determined by using a trypan blue exclusion assay. The cell pellet was thoroughly mixed with 350 µl (for < 5 x 10^6 cells) or 600 µl (for 5 x 10^6 – 1 x 10^7 cells) cell lysis buffer (RLT buffer) supplied with the RNeasy® Mini kit.

NanoDrop (Spectrophotometry)

The quality of extracted total RNA in wild-type and gemcitabine treated PANC-1 cells was examined using NanoDrop spectrophotometer. The concentration of total RNA extracted from wild-type PANC-1 cells was determined to be 118.8 µg/ml. The absorbance ratio at 260/280 nm was 2.106 which falls within the quality range of 2.1 – 1.8. However, the absorbance ratio at 260/230 nM was 1.650 which falls below the threshold of 1.8. Gemcitabine-treated PANC-1 cells (160 nM), yielded a higher RNA concentration reading by the NanoDrop of 242.8 µg/ml. This resulted in a good A260/280 ratio of 2.086 and a good A260/230 ratio of 2.176.

ABCC5 RT-qPCR

The fold change of ABCC5 160 nM was calculated to be 1065 (figure 3) suggesting that there is a large increase in expression and upregulation of ABCC5 with higher concentrations of gemcitabine.

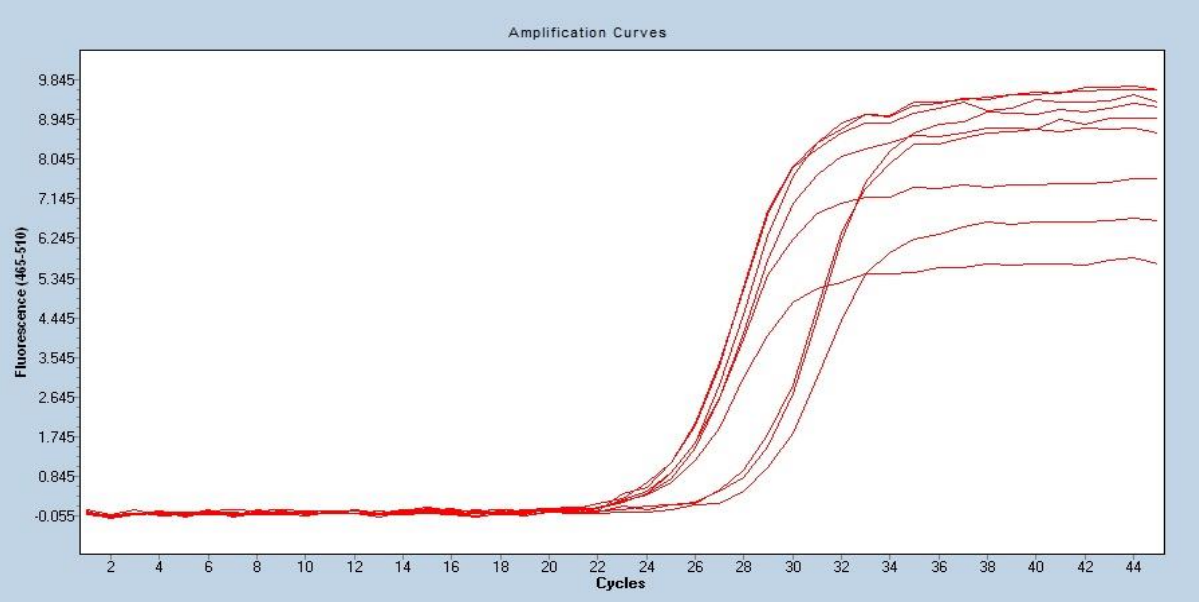
Figure 3: *Cp Values from qPCR*

	Cp Values (Duplicate)		
Sample Name	**1**	**2**	**3**
ABCC5 WT	24.88	24.94	24.66
ABCC5 160 nM	24.55	24.61	24.68

Sample Name	Mean of Cp Value (± SD)	ΔCp value	Variance of ΔCp/ΔΔCp value	Fold Change (2^-ΔΔCp)
ABCC5 WT	24.827 ± 0.147	6.773	0.154	1.000
ABCC5 160 nM	24.613 ± 0.065	-3.283	0.186	1065.021

(Figure 3: CP values obtained from qPCR results. Livak's formula is used to determine fold change.

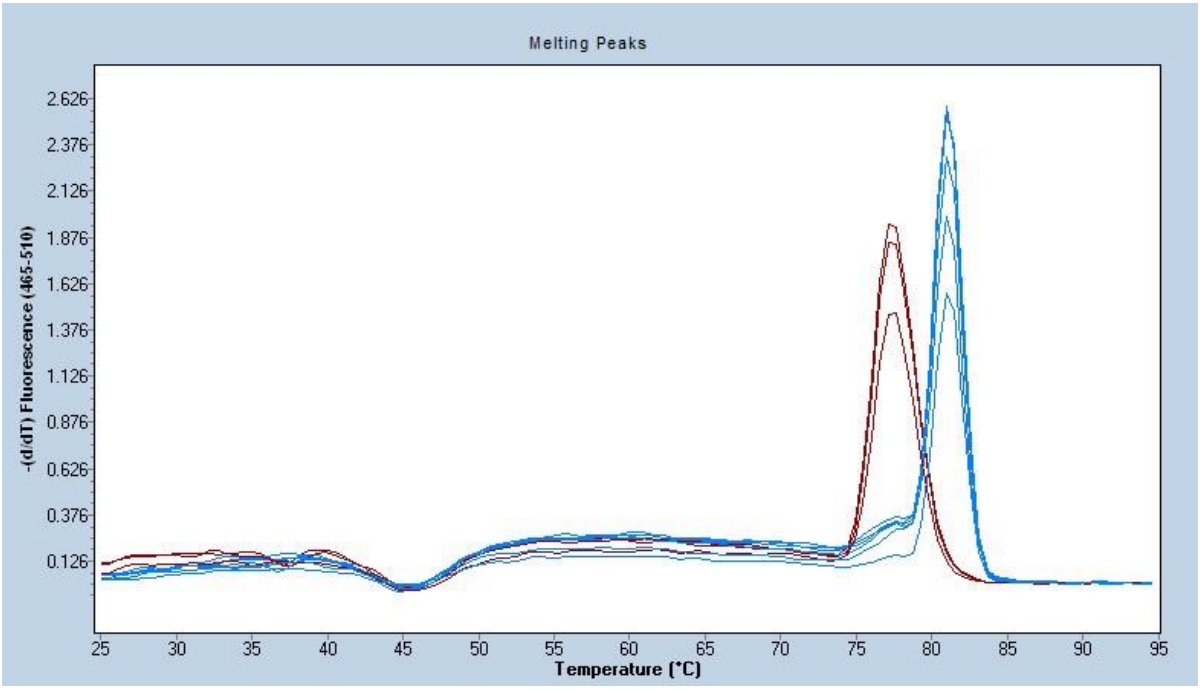
Figure 4: ***Relative Florescence vs Cycle Number***



(Figure 4): Relative fluorescence (465-510) vs cycle number. Amplification in semi-logarithmic view generated from serial dilutions of ABCC5.

ABCC5: Gene (Protein Coding), ATP Binding Cassette Subfamily C Member 5

Figure 5: ***Fluorescence vs Temperature***



(Figure 5): Fluorescence vs temperature graph of ABCC5 melting curve. The red lines indicate the wild-type peaks, and the blue lines indicate the homozygous ABCC5 melting peaks.

ABCC5: Gene (Protein Coding), ATP Binding Cassette Subfamily C Member 5

Discussion:

In pancreatic cancer cells, gemcitabine induces cytotoxicity by incorporating its active metabolites into the DNA chain and causing the termination of the synthesis pathway (Toschi et al., 2005). The cause for resistance to gemcitabine is multi-faceted and more research is required on possible mechanisms. This project focused on the generation of a gemcitabine-resistant pancreatic cell line and the regulation of ABCC5 transporters following selection with gemcitabine. The data presented in this paper suggest that resistance to gemcitabine in a widely available pancreatic cancer line may be attributed to an upregulation of the ABCC5 gene which leads to the over-expression of MRP5 transporter proteins on the cell membrane of tumor cells. An increase in the expression of ABC transporter proteins leads to an increase in the efflux of metabolites and xenobiotics, including gemcitabine, rendering chemotherapy treatment ineffective (Hagmann et al., 2010).

The ABCC5 (ATP Binding Cassette Subfamily C Member 5) gene encodes a 150 kDa MRP5 protein which belongs to ABC transporter protein superfamily. There are 7 families that ABC genes may fall into (MRP, MDR/TAP, White, GCN20, OABP, ALD, and ABC1). ABCC5 is a member of the MRP subfamily which confers multi-drug resistance. Tissue distribution of MRP5 is found to be ubiquitous including epithelial cells, smooth muscle cells, the blood-brain barrier, urethra, pyramidal neurons of the brain, and in pancreatic cells. Some model substrates for MRP5 include nucleotide analogs and cyclic nucleotides and MRP5 has been reported to confer gemcitabine resistance although the transport direction of gemcitabine by MRP5 has not been well characterised. Treatment with a limited number of anticancer drugs can often lead to the development of multidrug resistance (MDR). MDR is a mechanism that causes resistance to multiple therapeutic drugs, even when a patient is only treated with a few anticancer drugs. This resistance significantly reduces the effectiveness of chemotherapy

in treating pancreatic cancer. The MDR phenotype is frequently ascribed to the overexpression of certain ABC transporters. Overexpression of ABC transporters causes diminished drug accumulation into the target cells leading to a decreased available drug at the targets and thus increased drug resistance. ABC transporter-mediated drug efflux appears to be the major mechanism MDR development in a variety of cancers. One of the proposed mechanisms for ABC transporters is that they act as hydrophobic vacuum cleaners by extruding xenobiotics from the cells by utilising the driving force generated from ATP hydrolysis (Hagmann et al., 2010).

ABC Proteins

ABC transporters are comprised of two nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs) which make up the core unit of ABC transporters. The NBD is composed of highly conserved domains; the Walker A and Walker B sequences (the ABC signature motif), the H loop, and the Q loop, as well as several hydrophobic a-helices on the TMDs. Once ATP is binding with two NBDs, it is hydrolysed to form ADP and subsequently releases the conserved energy as the driving force for the transport function. ATP hydrolysis induces the conformational changes of the ABC transporter which allows the substrates bound to the TMDs to be released and extruded across the cell membrane. Currently, 48 ABC transporter genes have been functionally cloned in the human genome, organized into seven subfamilies from A to G (Jansen, R et al 2015).

Subfamily C of the ABC family (ABCC)

This family encompasses thirteen genes, one of which is the cystic fibrosis gene (CFTR, also known as ABCC7). The mutation of this gene results in an autosomal recessive disease

called cystic fibrosis. It is accepted that members of this family are linked to MDR. Furthermore, they have been found to possess additional functions which may contribute to the toxin excretion and metabolic syndromes. One of the major factors related to gemcitabine resistance in pancreatic cancer is genetic and/or epigenetic variations. Research has indicated mutations/dysregulation in the ABCC genes may result in multidrug resistance in various cancers including pancreatic cancer. This project outlines the findings of various investigations that have proposed that members of this family are implicated in pancreatic cancer being drug resistance. Because chemotherapeutic drugs are infused intravenously into the body, their disposition is complicated, such as their uptake and efflux into the cells, due to their complex pharmacokinetics and pharmacogenomics. As ABC transporters are expressed in the essential organs to deal with drug disposition and elimination, they can affect the pharmacokinetics of the executed chemotherapeutic agent. (Jansen, R et al 2015).

ABC transporters are accountable for actively transporting drug molecules out of the cells, by utilizing ATP as an energy source, which usually extrudes substances against the concentration gradient. This form of active transport decreases drug availability at the therapeutic targets and downgrades performance dramatically, causing chemotherapeutic failure in clinical management. Research has revealed that the ABC transporters are often overexpressed in malignant cells, which accordingly renders them more resistant to chemotherapeutic drugs; these ABC transporters portray a broad spectrum of drugs, containing P-glycoprotein (P-gp) substrates such as doxorubicin, docetaxel, and etoposide (Labbé, R et al., 2010).

Tumour cells with higher levels of P-gp are more resistant to drugs that act as P-gp substrates for this transporter. This phenomenon is referred to as P-gp-mediated multidrug

resistance (MDR) could be de novo or acquired, and it is neither due to acquired mutations nor influenced by mutational events. Instead, it seems to stem from increased expression of ABCB1 due to the fusion of a constitutively active promoter proximal to the transcription start site. Therefore, screening the expression levels of P-gp could be useful to select patients for a standard chemotherapy (Van Kalken *et al*, 1993). Additionally, research suggests that P-gp may also contribute to cell differentiation, migration, and invasion. It has been hypothesized that P-gp inhibitors could reverse MDR and improve the therapeutic efficacy of current standard regimens. However, the last three decades have seen the failure of the development P-gp inhibitors into anticancer drugs or an efficient member in the combination chemotherapies due to low efficacy and high toxicity (Fossaert et al, 2022).

Not all cancer types exhibit resistance due to P-gp overexpression and some anticancer drugs are not P-gp substrates. The involvement of other ABC transporters such as MRPs and BCRP has shifted the MDR paradigm. The MRP family consists of 13 members, ranging in size from 1325 to 1545 amino acids and encoded by the ABCC gene family. The second most significant efflux pump is MRP1/ABCC1 the MRP family that has the ability for drug efflux, which can efficiently extrude its drug substrates such as methotrexate and vincristine. Accumulating evidence suggests MRP1, and its overexpression have been associated with poor patient outcomes in many types of tumours, implicating that MRP1 plays an important role in tumor resistance. Clinical studies have revealed that the reversal of MRP1-mediated drug resistance seems to be more practical in comparison with targeting P-gp. Additionally, the over-expression of MRP5 in pancreatic cancer cells has been previously reported to confer resistance to nucleoside-based analogs. Therefore, further research in studying ABC transporters is required to improve chemotherapy and subsequently enhance the survival rate among patients.

MRP5 transports various organic anions including the anionic dye fluorescein diacetate, the cyclic nucleotides cGMP and cAMP, various nucleoside monophosphate analogs, glutamate conjugates, hyaluronan, and some glutathione S-conjugates. MRP5 has been suggested to gauge tissue levels of endogenous cyclic nucleotides and glutamate conjugates in a tissue-dependent manner. MRP5 substrates also include exogenous glutamate analogs, like the classic excitotoxic neurotoxins kainic acid, domoic acid, and N-methyl-D-aspartate (NMDA); the therapeutic glutamate analog ZJ43; and the anti-cancer drug methotrexate.

Currently, in humans, 48 ABC transporters genes have been recognised and classified into seven subfamilies. Specifically, members of ABCB/P-gp, ABCC, and ABCG subfamilies have been reported to be significantly involved in multidrug resistance in tumor cell lines. P-glycoprotein (P-gp), a member of the ABC-B family is one of the best-characterised ABC transporters; the physical structures of many other ABC transporters (especially MRP and BCRP) are based on P-gp's structure. In many cancer types, higher P-glycoprotein levels have been reported, and these higher levels have been found to be associated with MDR. Members of the ABCC family e.g. MRP1 (ABCC1), MRP3 (ABCC3), MRP4 (ABCC4), and MRP5 (ABCC5), have been found to be overexpressed in pancreatic tumours and are suspected to confer MDR in pancreatic cell lines *in-vitro* against a few chemotherapeutic drugs including gemcitabine and 5-FU. Due to the significant involvement of ABC transporters in MDR, it is believed that their inhibition could lead to more efficient results in the chemotherapy. Thus, over the time many inhibitors have been developed for ABC transporters, and their combinations with the drug have been tried to increase the drug efficacy (Jansen, R et al 2015).

The intracellular uptake of gemcitabine is mainly attributed to human equilibrative nucleoside transporter-1 (hENT1). In vitro studies and clinical evidence have demonstrated that hENT1 levels is positively correlated with the intracellular uptake of gemcitabine thus the efficacy of the gemcitabine. Changes in the activity of enzymes involved in gemcitabine metabolism can also negatively influence gemcitabine potency. For instance, CDA, deoxycytidine kinase (DCK) and ribonucleoside reductases M1 and M2 have been reported to confer gemcitabine resistance. MDR mechanism in pancreatic cancer may also involve abnormally expressed genes, altered signaling pathways, and unique tumor microenvironments, which have been illustrated using cell culture and animal models. In addition to standard chemotherapy, scanning for new molecular targets involved in drug resistance may overcome MDR and increase the efficiency of treatment in comparison with the single-agent regimen for a pancreatic tumor (Hagmann et al, 2012).

Members of the ABCC family are actively involved in pumping nucleoside-based drug analogs out of the cells, and their overexpression in pancreatic cancer cells induces MDR, and selection following drug treatment, in turn, enhances the protein abundance. Especially, functional overexpression of ABC proteins (MRP1, MRP3, MRP4, MRP5, and P-glycoprotein) was found in pancreatic cancer cells. MRP4 and MRP5 have also been localized in the duct cells, acinar cells, and pancreatic cells. Studies have also reported that MRP5 confers resistance to gemcitabine although there is no direct transport evidence at this stage. Thus, understanding the regulation of ABC transporter proteins could play a huge role in reversing MDR in pancreatic cancer. Studies have also reported variations in the MRP5 levels in the tumor grading correlated with the mRNA expression. Short-period treatment with gemcitabine alone or in combination with other chemotherapeutic drugs can cause several-fold induction in mRNA expression of ENT1 and MRP5, which are involved in gemcitabine

uptake and efflux, respectively (Hagmann et al, 2012). Whether and to what extent the induction in MRP5 and hENT1 levels remains unknown after the long-term selection by using gemcitabine. MRP5 and hENT1 are involved in the efflux of and uptake of gemcitabine and its moieties respectively. Two individuals from the multidrug resistance protein (MRP) family, MRP4, and MRP5 have been indicated to transport cAMP and cGMP. In the studies, it was found that MRP4 and MRP5 overexpressing cells show higher cGMP and cAMP efflux in an ATP-dependent manner. MRP5 is a low-affinity cyclic nucleotide transporter that acts as an efflux pump. MRP5 from the MRP family is confined in the plasma membrane and works as a transporter that pairs ATP hydrolysis to the efflux of organic anions. MRP5 was shown to transport the monophosphorylated type of nucleoside analog drugs (Mackey et al, 1999).

In a few studies, upregulation of ABC transporters has been reported in pancreatic cancer cells. Specifically, MRP3, MRP4, and MRP5 proteins were found to be up-regulated in human pancreatic carcinoma compared with normal pancreatic tissues. It has also been reported a higher mRNA level of ABCC5 in the pancreatic cancer cells as compared to normal pancreatic tissue. However, there is no clear evidence that the high level of ABCC5 is due to drug selection or innate capture of a constitutively active promoter. Nevertheless, the chemoresistance of pancreatic carcinomas towards multiple drugs is well-known. Such innate or acquired chemoresistance of an organ or cell sort can be because of induced expression and functional localisation of ABC transporter proteins. For example, the expression level of a few MRP isoforms has been shown to be affected by drugs or to contrast from typical conditions under pathophysiologic conditions. Gemcitabine is a deoxycytidine analog and its triphosphate metabolites inhibit DNA synthesis and induce apoptosis in pancreatic cancer, indicating the therapeutic targets are within pancreatic cancer cells. A few studies have demonstrated that a change in uptake transporters can intervene in resistance against these

drugs and metabolising enzymes. As we know now that gemcitabine is a prodrug and its active metabolites are dFdCDP and dFdCTP, and studies have demonstrated that nucleotide analogs and cyclic nucleotides are substrates for MRP4 and MRP5. Overexpression of one of these pumps may bring about resistance against anticancer and antiviral drugs. The MRP5 A-2G AA genotype also showed a significant association with overall survival in pancreatic cancer patients (log-rank P=.010) (Tanaka, et al, 2011).

MDR is an intricate process that can happen because of a few biochemical mechanisms that are still not comprehended: These are i) insufficient cellular drug accumulation caused by decreased drug uptake or by increased drug efflux; ii) perturbed expression of target enzymes or altered target enzymes; iii) diminished drug activation or accelerated degradation; iv) enhanced DNA repair; v) apoptosis escape. Some of these mechanisms may coincide, rendering the objective cell obstinate to treatment with drugs targeting a single target. The most commonly involved and principle mechanism of MDR is ABC transporter-mediated tumor resistance. Various in vitro cell models and animal studies have demonstrated that acquired resistance can be induced after initial differential responses. Usually, such an acquired resistance appears to be easily extended to the broad spectrum of anticancer drugs (Long et al, 2011).

The membrane transport proteins are categorised into four different subtypes: ion channels; co-transporters; aquaporins; and ATP-dependent pumps. Genes from each of the four classes are ancient, i.e., they can be found in most prokaryotes and almost all eukaryotes. The eukaryotic cell transporters are involved in the movement of amino acids, ions, sugars, and various xenobiotics in the cell and many cell organelles except nuclear membranes. Membrane transport proteins could be passive or active. When the substrates are

transported down the concentration gradient, they are called as passive transporters (aka uniporters or facilitative transporters) (Jansen, R et al, 2015).

Like ATP pumps, cotransporters intervene in coupled reactions, i.e., they are involved in the coupling of energetically unfavorable reaction to energetically favorable reactions. When the movement of transported and co-transported molecules is unidirectional across the cell membrane the transporter is known as a symporter; when vice versa, it is called an antiporter (or exchanger). If the intracellular net charge post-transport is negative than the procedure is named electronegative; if the net charge post-transport is positive than the procedure is electropositive. If the subsequent intracellular net charge stays unaltered, the procedure is named electroneutral.

Ion channels are pore-forming membrane proteins that assist in building and keeping up a slight voltage gradient across the cell membrane. Thus, ion channels are involved in the regulation of the cell's electric potential by permitting the ion flow down their electrochemical gradient. In general, ion channels are found in the closed state and have an extremely high efficiency of transport for cationic and anionic substrates. More than 400 genes are known for ion channel subunits. Ion channel transporters move ions at a slower rate than channel proteins. Aquaporins represent a unique class of transporter proteins, which mediate bi-directional water transport. In humans to date 13, putatively functional AQP genes have been reported (Hagmann et al, 2012).

ABC transporters are the ATP-dependent transmembrane efflux proteins which are involved in the import/export of various substrates in and out of the cells against their electrochemical gradient. These transporters are further divided as exporters and importers

based on their transport direction. Importers are responsible for the substrate uptake into the cells while the exporters are involved in the efflux (Hagmann et al, 2012).

Previous studies and literature support the evidence of increased expression of MRP5 in PANC-1 cells following drug treatment (Zhang et al., 2015). According to the cell viability counts, PANC-1 cells treated with 100 nM gemcitabine (Figure 2c) showed a small number of viable cells with a high number of observable apoptotic cells suggesting that an adequate concentration of gemcitabine was used to reach a therapeutic effect level, hence why, gemcitabine's mechanism of action was able to take place (Lu et al., 2019). At 2000 nM and 4000 nM, the cell viability increased (Figure 2d,2e). This may imply that at higher concentrations of gemcitabine treatment, PANC-1 cells acquired chemo-resistance (Lu et al., 2019).

To test the aim of this research, RNA extraction of ABCC5 was conducted in preparation for RT-qPCR. The wild-type untreated PANC-1 cells yielded a lower concentration of RNA according to NanoDrop results (118.8 µg/ml). The absorbance ratio at A260/230 (1.650) fell below the quality threshold (1.8) suggesting that there was a significant level of contaminants (particularly aromatic compounds) which have interfered with the reading. NanoDrop readings for the RNA extracted from the 160 nM gemcitabine-treated PANC-1 cells were higher, yielding 242.8 µg/ml of ABCC5. This is an increase of 124 µg/ml compared to the wild type. Absorbance ratios were within the respective quality ranges at both A260/280 and A260/230 which means there were no contaminants interfering with the readings (Labbé et al., 2010).

RT-qPCR results revealed that ABCC5 expression in gemcitabine-treated cell lines steadily increased when compared to wild-type untreated cell lines. These results are in

accordance with previous literature reports (You et al., 2018). The fold change in ABCC5 expression (1065) was calculated using Livak's method or the $2^{-\Delta\Delta Cq}$ method, using GAPDH as a housekeeping gene (Figure 3). This result was unexpectedly high and may be due to contaminant interference. Regardless, a positive fold change implies an upregulation of ABCC5 and an increase in the expression of MRP5 present on cell membranes (Hagmann et al., 2010). These results further reinforce the possible role of ABC transporter proteins in the acquired resistance to chemotherapy treatment.

Multi-drug resistance is one of the key causes of the failure of chemotherapy treatment (Long et al., 2011). Cancerous cells no longer respond to drugs and increasing the dose of administered medication is rendered ineffective at overcoming the acquired resistance (Grasso et al., 2017). As per the findings of this project, alternative methods need to be developed to overcome or bypass chemotherapy resistance. One method may be to use ABC transporter inhibitors which are selective for MRP5 while administering anticancer medication. In this case, it is important to administer ABC transporter protein inhibitors in a way that targets only those proteins located in pancreatic cells (Wijnholds et al., 2000). Otherwise, it would be necessary to use anti-cancer drugs which are not substrates for ABC transporter proteins such as 5-fluorouracil or alkylating drugs. These could even be used in combination with gemcitabine to improve therapeutic effects (Von Hoff et al., 2013).

Metastatic Cancer and Gemcitabine

The morbidity rate for patients in the metastatic stage of pancreatic cancer is significantly high, and chemotherapy is the primary option for these individuals. For those whose disease is unresectable, metastatic disease radiation combined with chemotherapy may be chosen as an alternate. Nevertheless, the outcomes of either treatment are only slightly effective in increasing the survival rate and reducing cancer-related symptoms.

Combination chemotherapy, which has better results, can be used by clinicians with a good Performance Status (PS). It is therefore possible for PDAC patients to receive mixed or single-agent treatment depending on their PS. While multidrug regimens may increase the anti-tumor response of a patient, they are also risks associated with higher toxicity and adverse health effects. Even though most therapeutic regimens have some intricacies, such as reduced blood cell counts, vomiting and diarrhoea, nausea, mouth ulcers, constipation, poor appetite, nervous system changes, hair loss, and infertility. Side effects, like blood clotting and weight loss, are one of the reasons forcing patients to their early termination due to the less efficacy of these treatments. Gemcitabine was identified in 1997 as a potential first-line treatment for patients in good health. Several studies have highlighted its superiority over 5-FU, the most visible being a phase III study involving 126 participants, with 23.8% of gemcitabine-treated patients displaying a clinical benefit response compared to 4.8% of those given 5-FU. Unfortunately, certain side effects as mentioned above have hindered these treatments' effectiveness and led to their discontinuation. The mean survival time for patients receiving gemcitabine and 5-FU-based treatment was 5.6 and 4.4 months respectively, with the one-year survival rate for gemcitabine being 18% and for 5-FU being 2%. Gemcitabine has been seen to improve the symptoms of disease in those who received it. Furthermore, a stage II/III trial revealed positive or fractional positive responses to gemcitabine which ranged from 5.4% to 12%, along with an overall median survival time between 5 to 7.2 months. Another study recorded a one-year survival rate of 18% paired with a median survival time of 6.2 months. The only side effect seen so far has been grade 3 and 4 myelosuppression in around 30% of patients, but overall, it is noted that the systemic toxicity associated with this regimen is relatively low. (Klein, B et al., 2000).

Conclusion:

Multi-drug resistance is one of the key causes of chemotherapy failure and is becoming a central issue with cancer treatment. The results of this research suggest that higher concentrations of gemcitabine treatment induce chemoresistance in PANC-1 cells. As can be seen from the cell viability results, which show that at a concentration of 2000 nm and 4000 nm, gemcitabine treatment was rendered ineffective at causing cancer cell apoptosis. These results are in accordance with findings from previous studies. The RT-qPCR results show a fold change of 1065. A fold change value of 1065 was unexpectedly high and may be due to interference or contamination of the sample. Regardless, the value strongly suggests that ABCC5 was upregulated and showed high expression as gemcitabine concentrations increased. The up-regulation of ABCC5 may have contributed to the acquired resistance of gemcitabine treatment therefore it may be of importance to do further research into down-regulating ABCC5 expression to maintain therapeutic chemotherapy cytotoxic effects. Patients who relapse after treatment with MDR drugs often display an MDR phenotype. It may be of clinical interest to select patients based on ABCC5 expression and screening for overexpression of P-gP or MRP1 as a predictor of gemcitabine responsiveness should be considered for standard chemotherapy for pancreatic cancer (Van Kalken *et al*, 1993).

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